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TITLE: Effects of Csk Homologous Kinase Overexpression  
on HER2/Neu-Mediated Signal Transduction Pathways  
in Breast Cancer Cells

PRINCIPAL INVESTIGATOR: Radoslaw Zagozdzon, M.D., Ph.D.  
Hava Avraham, Ph.D.

CONTRACTING ORGANIZATION: Beth Israel Deaconess Medical Center  
Boston, MA 02115

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13. ABSTRACT (Maximum 200 Words)  Our proposal aimed to investigate the potential function of CHK as a signal transducer in the signaling pathway from HER2/Neu receptor. Our major accomplishments are as follows: 1. a. We described the effects of CHK on the HER2/Neu signal transduction towards RAFTK and Akt kinases. b. We have identified modulation by CHK of CXCR4 expression in breast cancer cells. 2. a. We described the correlation of the binding affinity of CHK to HER2/Neu with the effectiveness of the inhibition of Src kinase by CHK. b. We evaluated the role of CHK in the development of Neu-induced mammary tumors in transgenic mice. 3. We generated CHK protein derivatives and evaluated their usefulness as potential tools for protein therapy of breast cancer in vitro. Our results confirm the notion that CHK is a signal transduction modulator HER2/Neu-mediated pathway in breast cancer cells. However, the effects elicited by CHK are ambiguous. CHK is capable of eliciting both inhibitory and stimulatory effects in breast cancer cells. The results obtained in transgenic mouse model do not reaffirm the potential role of CHK in formation of Neu-induced mammary carcinomas. However, in the inhibitory effects of CHK suggest potential role of CHK in breast cancer invasion and metastasis. These findings warrant further investigations.				
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Final Report for Award # DAMD17-02-1-0302

**Title: EFFECTS OF CSK HOMOLOGOUS KINASE OVEREXPRESSION ON HER2/NEU-MEDIATED SIGNAL TRANSDUCTION PATHWAYS IN BREAST CANCER CELLS.**

**P.I.: RADOSLAW ZAGOZDZON, M.D., PH.D.**

**INTRODUCTION:** The onset of 90% of all breast cancers is random and spontaneous, while 10% of cancers have been linked to specific mutations in autosomal dominant breast cancer susceptibility genes such as BRCA1 and BRCA2. Random onset of breast cancers has in many cases been correlated with increased HER2/Neu (also termed ErbB-2) expression and Src family tyrosine kinases activity. The exact molecular mechanism of this phenomenon remains unknown. The initial steps in HER2/Neu pathways are complex, and are modulated by such processes as the autophosphorylation, cross-phosphorylation and dimerization of ErbB molecules. Furthermore ErbB cascades also interact with other signaling pathways. Association of c-Src with these receptor tyrosine kinases (PTKs) is an integral part of the signaling events mediated by the receptors, and may contribute to the malignant transformation of cells. Increased Src kinase activity observed in HER2/Neu-induced tumors results from the ability of the Src-SH2 domain to directly interact with HER2/Neu in a tyrosine phosphorylation-dependent manner. Since HER2/Neu and pp60src play a role in breast cancer and are altered during malignant transformation and tumor progression, it is important to characterize the regulation of these protein kinase activities, and the likely interactions of these kinases with each other.

Src family kinase activity is inhibited by the phosphorylation of a conserved, carboxy-terminal tyrosine. The protein tyrosine kinase responsible for this phosphorylation is Csk. We and others identified a second member of the Csk family – Csk Homologous Kinase (CHK). CHK has been suggested to have a specific role in breast cancer and the potential to be a target of breast cancer drug development. CHK, which is specifically expressed in primary breast cancer specimens, but not in normal breast tissues, phosphorylates Src and down-regulates its activity. Previous biochemical data also suggested that CHK acts as a negative growth regulator of human breast cancer. Furthermore, the interaction between the CHK-SH2 domain and pTyr<sup>1248</sup> of the HER2/Neu receptor is specific and critical for CHK function.

**BODY:**

The results mentioned above lead us to hypothesize that: (i) CHK is able to antagonize the growth-promoting signals that are mediated by HER2/Neu and Src kinases; (ii) enhancement of the binding affinity of CHK to the HER2/Neu protein might further increase the antitumor effects of CHK in breast cancer cells; (iii) peptide derived from an enhanced-binding mutant of CHK may retain its inhibiting ability on HER2/Neu and Src-mediated signaling. Therefore, the goals of this project are: (1) to investigate the effects of CHK on downstream signaling from the HER2/Neu receptor; (2) to assess the possibility of enhancing the inhibitory effects of CHK on HER2/Neu-mediated signaling; (3) to test the anti-tumor effects of CHK-derived peptides designed to diminish the transformation potential of HER2/Neu.

## **Task 1**

### **Year 1:**

**a.** Our recent observations indicated that RAFTK (also termed PYK2 and CAK- $\beta$ ) participated in MAPK-mediated intracellular signaling upon heregulin (HRG) stimulation and promoted breast carcinoma invasion. Analyses of the members of the HRG-stimulated complex revealed that RAFTK is associated with p190 RhoGAP (p190), RasGAP (modifying the MAPK transduction pathway) and ErbB-2, and plays an essential role in mediating the tyrosine phosphorylation of p190 by Src. Furthermore, studies from our group indicate that the Csk homologous kinase (CHK), a member of the Csk family, directly associates with HER2/Neu and down-regulates HER2/Neu-mediated Src kinase activation in breast cancer cells upon heregulin stimulation. Since activation of RAFTK is associated with the activity of Src family kinases, we analyzed whether CHK is capable of opposing HRG-induced activation of RAFTK. Stimulation of human T47D breast cancer cells with HRG induced the tyrosine phosphorylation of RAFTK and its association with CHK in vitro and in vivo. This interaction was mediated through the Src binding site (amino acid residue at 402) of RAFTK and the SH2 domain of CHK. RAFTK phosphorylation downstream of the activated HER2/Neu was greatly reduced in the presence of CHK. Maximal inhibition of RAFTK phosphorylation by CHK required the kinase activity of CHK. Furthermore, CHK inhibited the tyrosine phosphorylation of the focal adhesion-associated protein, paxillin, and inhibited HRG-induced T47D breast cancer cell migration. These findings indicate the role of CHK as a negative regulator in HRG- and RAFTK-mediated intracellular signaling in breast cancer cells (for details please refer to Attachment #1).

**b.** The serine/threonine kinase Akt has recently been the focus of intense research. Akt activation requires the phosphorylation of both Thr-308 and Ser-473. Src kinase was shown to induce activation of Akt, while Lyn kinase seems to inhibit this activation. In the present study, we investigated the effect of overexpressing the Csk homologous kinase (CHK), an inhibitor of Src-family kinases, on the phosphorylation of Akt induced by two different factors: heregulin or cisplatin. We used MCF-7 cells stably overexpressing the wild-type CHK [CHK(wt)] or dead-kinase CHK [CHK(dk)]. We observed that in MCF-7 CHK(wt) cells Lyn kinase activity was more profoundly inhibited than Src kinase activity. When the cells were stimulated with heregulin or cisplatin, Akt phosphorylation occurred more rapidly in MCF-7 CHK(wt) cells in comparison to the other clones used. Interestingly, MCF-7 CHK(wt) cells in vitro were markedly more resistant to cisplatin than the other clones used in the experiments, and surprisingly chemical inhibition of Akt phosphorylation did not influence this resistance. In summary, our results show facilitation of Akt phosphorylation by the overexpression of CHK, and provide new insight into the putative role of CHK in human cancer. (for details please refer to Attachment #2)

### **Year 3:**

During our studies on the effects of CHK on the signal transduction from the HER2/Neu receptor, we have performed a microarray gene analysis in breast cancer cells overexpressing CHK. We have found a significant regulation of CXCR4 expression by CHK. Because CXCR4 has recently been suggested to play role in breast cancer invasion

and metastasis, we have pursued these investigations. In these studies, we have found that CHK kinase activity is involved in the regulation of CXCR4 by altering YY1 binding to the CXCR4 promoter. Whereas CHK had no significant effects on the expression of YY1, c-Myc, Max, and other YY1-binding proteins, CHK was found to modulate the YY1/c-Myc association. Furthermore, CHK inhibited CXCR4-positive breast cancer cell migration. Taken together, these studies show a novel mechanism by which CHK down-regulates CXCR4 through the YY1 transcription factor, leading to decreased CXCR4-mediated breast cancer cell motility and migration. Please find the detailed results of these investigations in the publication attached as an Attachment # 3.

## **Task 2**

### **Year 1:**

The interaction between the CHK SH2 domain and Tyr(P)(1248) of the ErbB-2 receptor has been shown to be specific and critical for CHK function. In our studies, we investigated whether the interaction of the CHK SH2 domain and ErbB-2 is directly related to the inhibition of heregulin-stimulated Src kinase activity. We constructed three CHK SH2 domain binding mutants: G129R (enhanced binding), R147K (inhibited binding), and R147A (disrupted binding). NMR spectra for the domains of each construct were used to evaluate their interaction with a Tyr(P)(1248)-containing ErbB-2 peptide. G129R showed enhanced binding to ErbB-2, whereas binding was completely disrupted by R147A. The enhanced binding mutant showed chemical shift changes at the same residues as wild-type CHK, indicating that this mutant has the same binding characteristics as the wild-type protein. Furthermore, inhibition of heregulin-stimulated Src kinase activity was markedly diminished by R147A, whereas G129R-mediated inhibition was stronger as compared with wild-type CHK. These results indicate that the specific interaction of CHK and ErbB-2 via the SH2 domain of CHK is directly related to the growth inhibitory effects of CHK. These new CHK high affinity binding constructs may serve as good candidates for inhibition of the ErbB-2/Src transduction pathway in gene therapy studies in breast cancer (for details please refer to Attachments #4).

### **Years 2 and 3:**

To study the effects of CHK on ErbB2-induced tumorigenesis in breast in vivo, we initially proposed injections of the stable CHK-transfected breast cancer cell lines into nude mice. However, the advent of transgenic mice now permits manipulation of mouse genome with the aim of studying the involvement of particular genes in tumorigenesis and disease progression and of developing mouse models of human genetic disease. The utility of such technologies is emphasized in transgenic mice expressing genes thought to play important roles in the initiation and progression of mammary carcinomas. Therefore, as reported in the annual report covering the period April 1, 2003- March 31, 2004, we have decided to employ an alternative and more nature-related model of mammary tumorigenesis in vivo, namely the mammary-tissue specific transgenic model. This decision was approved by the Reviewers of the report. In this report, we summarize our results achieved in Years 2 and 3 in an attached manuscript (please see Attachment # 5).

### Task 3

#### **Year 2:**

“Protein therapy” is a newly developed method which allows proteins, peptides and biologically active compounds to penetrate across the plasma membrane of eukaryotic cells via homopolymers or peptides containing a high percentage of cationic amino acid protein transduction domains (such as polyarginine, 9-12-arginine peptide tag) (1, 2). This method enables us to control the localization of targeted substances in subcellular compartments, such as the nuclei, mitochondria and post-synaptic density. The method is very efficient and applicable not only to cultured cells but also to tissue slices and the whole animal. Brain, heart, skeletal muscle, liver, pancreas and lymphocytes are efficient target organs and tissues for protein therapy (for review see (3)).

In our project we proposed to study the effects of CHK-derived peptides fused with polyarginine on Neu signaling in breast cancer cells. However, based on our NMR studies of SH2 domain of CHK, recently published and reported in previous annual report, we disqualified the primarily designed peptides from further studies. Our studies suggested that whole SH2 domain of CHK instead of particular peptides is necessary for this protein to interact with activated Neu (Kim S., personal communication). Thus, we chose an alternative approach, and designed full-length CHK-based polyarginine-fusion protein derivative as a research tool and potential therapeutic agent.

Generation of a CHK-based polyarginine-fusion protein derivative as a research tool and potential therapeutic agent: Introduction of polyarginine fusion proteins into mammalian cells: To initially estimate the uptake of polyarginine fused protein into T47D breast cancer cells, we introduced cDNA encoding nine consecutive arginines into pGEX4T-1 vector and then expressed and purified the GST-9Arg fusion protein in the E. coli expression system. We estimated the uptake of this protein by PC12 cells in-vitro. As shown in Appendix 6, Fig. 1, incubation of cells for 30 min with GST-9Arg protein concentrations as low as 4 µg/ml resulted in the appearance of the specific protein band in the total cell lysate when assessed by Western blotting with anti-GST antibody. Another construct of this type created in our lab is EGFP-12Arg fusion protein. Our time point experiments show that when cells are incubated in the presence of GST-9Arg or EGFP-12Arg fusion proteins, each of these proteins can be detected within the cells without apparent toxicity continuously for at least 24 or 72 hours, respectively (data not shown).

*Generation of expression vectors encoding CHK-polyarginine fusion protein:* Recently, transduction of an active enzyme, human catalase, mediated by arginine-rich peptides into mammalian cells, was reported (4). Based on these results, we attempted to generate a CHK-polyarginine derivative as a potential tool to study the role of CHK in breast cancer. Our first choice was to express full-length CHK-polyArg fusion protein in the bacterial expression system. Unfortunately, our experiments indicate that CHK expression in E. coli undergoes excessive proteolytic degradation within the kinase domain, which results in dramatically little yield (data not shown). Our observations were confirmed by a recent publication by Ayarpetov et al. (5). Therefore, we chose a mammalian expression system instead to produce and subsequently purify CHK-polyArg protein. Additionally, we tagged the C-terminus of CHK with GFP, to enable visual control of the protein uptake and subsequent subcellular localization. Schematic maps of the generated vectors (control GFP-12Arg and CHKwt/dk-GFP-12Arg) are presented in

Fig. 2A. We analyzed the expression of newly generated proteins after transient transfection of CHO cells by Western blotting using anti-CHK and anti-GFP antibodies (Fig. 2B). We also assessed the transiently transfected CHO cells under a fluorescent microscope. As shown in Fig. 3, all proteins containing 12Arg tended to densely localize in the nucleoli. This fact is not surprising, because Arg is a cationic amino acid and, when polymerized, is capable of binding anionic structures, such as nucleic acids. This capability was recently used in non-viral gene delivery techniques (for review see (6)). However, while GFP-12Arg protein showed a widely diffuse localization within the transfected cells (Fig. 4A), both CHKdk and CHKwt localized mostly within the cytoplasm (Fig. 4B and 4C, respectively). This strongly suggested the potential usefulness of our newly generated constructs in studies on the subcellular localization of CHK and co-localization with other proteins.

Some disadvantage of the mammalian expression system to obtain purified proteins is a small yield, which could slow down our studies. Therefore, we attempted to create another CHK derivative, which can be expressed in bacteria. To this end, we fused SH3 and SH2 domains of CHK with kinase domain of Csk.

Construction of CHK/Csk (CHSK) chimeric fusion cDNA. To generate CHK/Csk (CHSK) chimeric fusion cDNA, an Xba I restriction site was introduced by PCR into both cDNA sequences in the region between SH2 and kinase domains (Fig. 5).

Then, both PCR products were digested with appropriate restriction enzymes, ligated pIRES2-EGFP plasmid (Clontech) with the use of Nhe I and Xho I enzyme sites. Ligation product was evaluated by restriction enzyme analysis, sequencing, and transient transfection of 293T followed by Western Blot (Fig. 11A). Enzymatic activity of newly generated CHSK chimeric protein was assessed using in vitro tyrosine kinase assay (Fig. 11B, C), as described previously (7).

For further analysis of CHSK chimeric protein we attempted to express and to purify CHK, Csk and CHSK as GST-fusion proteins in *E. coli* expression system. In contrast to CHK, CHSK and Csk were successfully expressed in this system retaining their kinase activities after purification (data not shown).

### **Year 3:**

As reported in the annual report for Year 2 of our studies (please see above), we have generated: 1. CHK-12Arg fusion protein as well as 2. CHSK chimeric protein.

1. CHK-12Arg fusion protein – although CHK-12Arg was easily detected in the transfected cells (Fig. 2) by Western blotting, this method of protein expression did not yield an amount of protein sufficient for application for protein therapy purposes.
2. Having the results of increased kinase activity of CHSK in comparison with CHK (Fig. 5), we attempted to verify if the CHSK chimeric protein binds the activated form HER2/Neu. To this end, we have generated bacterial-expression pGEX4T-1-based construct expressing GST-CHSK fusion protein. The GST-CHSK fusion protein was expressed and purified as described previously (8). Fig. 6 shows SDS-PAGE analysis followed by Coomassie staining of a GST-CHSK fusion protein expressed in *E. coli* and bound to glutathione-Sepharose beads. As a positive



control for binding of CHK to phosphorylated HER2/Neu, we have used SH2 domain of CHK expressed in *E. coli*, as described previously (8). The GST pull-down assay was performed as described previously (8) using the protein lysate of heregulin-stimulated MCF-7 cells. As expected, SH2 domain of CHK pulled down phosphorylated form of HER2/Neu. However we did not observe association of CHSK with HER2/Neu. It is our assumption that the structural manipulation introduced to the CHK protein by fusing its SH3 and SH2 domains with kinase domain of CHK has hampered the ability of SH2 domain to bind activated HER2/Neu. Thus, we have not been able to use CHSK chimeric protein for further studies.

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#### KEY RESEARCH ACCOMPLISHMENTS:

Our major accomplishments are as follows:

##### Task 1:

- a. We have described the effects of CHK on the HER2/Neu-mediated signal transduction towards RAFTK and AKT kinases.
- b. We have also identified modulation by CHK of CXCR4 expression and function in breast cancer cells.

##### Task 2.

- a. We have described the correlation of the binding affinity of CHK to HER2/Neu with the effectiveness of the inhibition of Src kinase by CHK.
- b. We have evaluated the role of CHK in the development of Neu-induced mammary tumors in genetically modified mice.

Task 3. We have generated CHK protein derivatives and evaluated their usefulness as potential tools for protein therapy of breast cancer in vitro.

## REPORTABLE OUTCOMES:

So far we published 4 original papers as a result of our project:

1. McShan, G.D., R. Zagozdzon, S.Y. Park, et al., *Csk homologous kinase associates with RAFTK/Pyk2 in breast cancer cells and negatively regulates its activation and breast cancer cell migration*. Int J Oncol, 2002. 21(1): pp. 197-205. (Attachment #1)
2. Zagozdzon, R., C. Bougeret, Y. Fu, et al., *Overexpression of the Csk homologous kinase facilitates phosphorylation of Akt/PKB in MCF-7 cells*. Int J Oncol, 2002. 21(6): pp. 1347-52. (Attachment #2)
3. Kim, S., R. Zagozdzon, A. Meisler, et al., *Csk homologous kinase (CHK) and ErbB-2 interactions are directly coupled with CHK negative growth regulatory function in breast cancer*. J Biol Chem, 2002. 277(39): pp. 36465-70 (Attachment #4)
4. Lee, B.-C., T.-H. Lee, R. Zagozdzon, et al., *Carboxyl-Terminal Src Kinase Homologous Kinase Negatively Regulates the Chemokine Receptor CXCR4 through YY1 and Impairs CXCR4/CXCL12 (SDF-1a)-Mediated Breast Cancer Cell Migration*. Cancer Res, 2005. 65(7): pp. 2840-45 (Attachment #3)

Currently, we have prepared a manuscript entitled: *Csk-homologous kinase (CHK) – the role in mammary development and tumorigenesis in transgenic mouse model*, which will be submitted in the nearest future. Please find it attached as an Attachment #5.

## LIST OF PERSONNEL RECEIVING PAY FROM THE RESEARCH EFFORT:

Radoslaw Zagozdzon

## CONCLUSIONS:

Our results confirm the notion that CHK is a signal transduction modulator following activation of HER2/Neu receptor in breast cancer cells. However, the effects elicited by CHK in breast tissue are ambiguous. According to our results, CHK is capable of eliciting both inhibitory (inhibition of Src and RAFTK activation, downregulation of CXCR4) as well as stimulatory (facilitation of Akt activation in vitro, activation of MAPK pathway in vivo) effects in breast cancer cells. Moreover, our results obtained in transgenic mouse model do not reaffirm the potential role of CHK in formation of Neu-induced mammary carcinomas. However, effects of CHK on Src- and RAFTK-mediated signaling as well as the negative regulation of CXCR4 function in breast cancer cells suggest potential role of CHK in breast cancer invasion and metastasis. These findings warrant further investigations.

## Csk homologous kinase associates with RAFTK/Pyk2 in breast cancer cells and negatively regulates its activation and breast cancer cell migration

GINA D. McSHAN<sup>1</sup>, RADOSLAW ZAGOZDZON, SHIN-YOUNG PARK, SHEILA ZRIHAN-LICHT, YIGONG FU, SHALOM AVRAHAM and HAVA AVRAHAM

Division of Experimental Medicine, Beth Israel Deaconess Medical Center, Harvard Institutes of Medicine, 4 Blackfan Circle, Boston, MA 02115, USA

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**Abstract.** Our recent observations indicated that RAFTK (also termed Pyk2 and CAK- $\beta$ ) participated in intracellular signaling upon heregulin (HRG) stimulation and promoted breast carcinoma invasion. Furthermore, studies from our group indicate that the Csk homologous kinase (CHK), a member of the Csk family, directly associates with HER2/Neu and down-regulates HER2/Neu-mediated Src kinase activation in breast cancer cells upon heregulin stimulation. Since activation of RAFTK is associated with the activity of Src family kinases, we analyzed whether CHK is capable of opposing HRG-induced activation of RAFTK. Stimulation of human T47D breast cancer cells with HRG induced the tyrosine phosphorylation of RAFTK and its association with CHK *in vitro* and *in vivo*. This interaction was mediated through the Src binding site (amino acid residue at 402) of RAFTK and the SH2 domain of CHK. RAFTK phosphorylation downstream of the activated HER2/Neu was greatly reduced in the presence of CHK. Maximal inhibition of RAFTK phosphorylation by CHK required the kinase activity of CHK. Furthermore, CHK inhibited the tyrosine

phosphorylation of the focal adhesion-associated protein, paxillin, and inhibited HRG-induced T47D breast cancer cell migration. These findings indicate the role of CHK as a negative regulator in HRG- and RAFTK-mediated intracellular signaling in breast cancer cells.

### Introduction

Focal adhesion and dynamic changes in actin cytoskeleton are involved in cell growth, shape and in tumor invasion. Recently, a new family of protein tyrosine kinases (including FAK and RAFTK) was identified as the focal adhesion kinase family (reviewed in ref. 1). FAK is a widely expressed non-receptor tyrosine kinase, which participates in integrin-mediated signal transduction and regulatory pathways governing cellular functions such as adhesion, motility, anchorage-independence, and the G1 to S phase transition of the cell cycle (2-4). RAFTK (also known as Pyk2 and CAK- $\beta$ ) shares 48% homology with FAK, but lacks transmembrane and myristylation domains, as well as SH2 and SH3 domains (5). Like FAK, RAFTK possesses a predicted proline-rich region in its C-terminal. RAFTK is implicated, along with pp60 src, in the coupling of some G-protein linked receptors to the MAP kinase pathway (6). RAFTK also participates in stress signaling and the JNK pathway (7), as well as in T-cell receptor signaling (8), integrin-dependent and independent signaling (9-12), and megakaryocyte signal transduction (1,12).

Expression of FAK and RAFTK was observed in breast cancer cells (13). We also observed that RAFTK is tyrosine-phosphorylated upon HRG stimulation in these cells, while FAK is constitutively tyrosine-phosphorylated in the cells (13). More importantly, our recent observations indicate that RAFTK-mediated intracellular signaling upon HRG stimulation can promote breast carcinoma cell invasion (13).

Activation of RAFTK seems to rely on autophosphorylation of Tyr-402, with subsequent association with Src family kinases, which phosphorylate other tyrosine residues within RAFTK (6). The Src family of cytoplasmic tyrosine kinases plays a critical role in proliferation, cell-cell interaction, cytoskeletal organization and signaling. Src family kinase activity is inhibited by phosphorylation of a conserved carboxyl-terminal tyrosine (Tyr-527) (14). Mutation of this conserved

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**Correspondence to:** Dr H. Avraham, Division of Experimental Medicine, Beth Israel Deaconess Medical Center, Harvard Institutes of Medicine, 4 Blackfan Circle, Boston, MA 02115, USA  
E-mail: havraham@caregroup.harvard.edu

**Present address:** <sup>1</sup>Fox Chase Cancer Center, Department of Medical Oncology, 7701 Burholme Avenue, Room W 260, Philadelphia, PA 19111, USA

**Abbreviations:** CHK, Csk homologous kinase; EGF, epidermal growth factor; FAK, focal adhesion kinase; GAP, GTPase activating protein; HRG, heregulin; MAP, mitogen-activated protein; PAK1, p21 activated kinase 1; PI-3 kinase, phosphatidylinositol-3 kinase; RAFTK, related adhesion focal tyrosine kinase; SH1, Src homology 1 domain; SH2, Src homology 2 domain; SH3, Src homology 3 domain; Tc, tetracycline

**Key words:** breast cancer, migration, CHK, RAFTK/Pyk2, heregulin

tyrosine residue constitutively activates c-Src and renders it oncogenic (14-16). Csk, C-terminal Src kinase, phosphorylates the conserved carboxyl-terminal tyrosine (Tyr-527) of c-Src, suppressing c-Src kinase activity (17-23). Csk has been shown to be involved in the negative regulation of the kinase activity of Src family members *in vivo* (24,25). We and several groups identified a second member of the Csk family, known as CHK (Csk homologous kinase) (26-31). Both Csk and CHK kinases phosphorylate the conserved inhibitory tyrosine of Src family kinases, thereby repressing their kinase activity. CHK was comparable to Csk in its ability to down-regulate the *in vivo* activity of the Src family kinases, Fyn and c-Src (32-34).

Previously, we have demonstrated a specific interaction of CHK with the HER2/Neu growth factor receptor, mediated by the SH2 domain of CHK (35-37). This interaction was recently found to be mediated by Tyr 1253, the autophosphorylation site of rodent Neu homologous to the site Tyr 1248 in human HER2/Neu. This site confers oncogenicity and transforming abilities to the receptor (38-40). Additionally, CHK down-regulated the ErbB-2-mediated activation of Src family tyrosine kinases and elicited an anti-proliferative effect (38). The involvement of CHK in the ErbB-2-mediated signaling pathway led us to investigate whether CHK participates in heregulin (HRG)-induced breast cancer cell migration and whether it is capable of modulating the RAFTK-mediated signal transduction.

In this study, we demonstrated a novel interaction between CHK and RAFTK upon HRG stimulation in breast cancer cells. This interaction was mediated through the Src-binding site (amino acid residue at 402) of RAFTK and the SH2 domain of CHK. We also demonstrated that CHK inhibited the tyrosine phosphorylation of RAFTK and its catalytic activity in response to HER2/Neu-mediated signaling. Transfection studies revealed that in RAFTK-expressing breast cancer cells, CHK inhibited HRG-induced cell migration. Taken together, these results suggest a novel role for CHK as a negative regulator of RAFTK during HRG-induced signaling in breast cancer cells.

## Materials and methods

**Materials.** Recombinant human HRG (HRG-B1, 177-244) was generously provided by Dr Mark A. Sliwkowski, Genentech (San Francisco, CA). Polyclonal antibodies to CHK (Lsk), RAFTK polyclonal antibodies (RAFTK), goat anti-Pyk2, and GST antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal phosphotyrosine antibody (4G10) was kindly provided by Dr Brian Druker (Oregon Health Sciences Center). Monoclonal phosphotyrosine antibody (PY20) was purchased from Transduction Laboratories (Lexington, KY). M2 monoclonal antibodies specific for the Flag epitope were obtained from Sigma. Reagents for electrophoresis were obtained from Bio-Rad (Hercules, CA). Chemiluminescent reagents were obtained from NEN (Boston, MA). All other reagents were purchased from Sigma (St. Louis, MO).

**Cell lines and transfection.** Human embryonic kidney cells (293 cells) and breast cancer cells (T47D) were obtained

from ATCC (American Type Culture Collection, Rockville, MD). 293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. T47D cells were maintained in RPMI media with 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were transfected by the calcium phosphate method.

**DNA constructs.** Plasmids expressing Flag-epitope-tagged CHK (pcDNA3 Flag-CHK), pcDNA3 Flag-dead-kinase CHK (dkCHK), pcDNA3 Flag-RAFTK, Flag-RAFTK Y402F were described previously (25,34,38,40,41).

**Production of GST-fusion proteins and precipitations.** GST-fusion proteins of SH3-SH2-CHK, SH2-CHK, SH3-CHK, and the GST control protein were cloned into the GST-fusion expression vector pGEX (Amersham Pharmacia Biotech), and proteins were expressed and purified as previously described (36,37). Precipitation experiments used 1 mg of cell lysate and 10  $\mu$ g of the indicated GST-fusion proteins.

**Immunoprecipitations and antibodies.** Cells,  $5 \times 10^6$  293 or T47D, were transfected using the calcium phosphate method and were lysed 48-h post-transfection with modified RIPA buffer containing protease inhibitors (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM NaF, 1 mM  $\text{Na}_2\text{VO}_4$ , and 1 mM each aprotinin, leupeptin, and pepstatin). Immunoprecipitations were performed using polyclonal rabbit CHK-specific antibodies (anti-Lsk) or polyclonal goat RAFTK-specific antibodies (anti-Pyk2). Control antibodies for the immunoprecipitations were, respectively, rabbit preimmune serum (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-GST polyclonal goat antibodies (Santa Cruz Biotechnology). Immunoprecipitation products were analyzed by SDS-PAGE, transferred to nitrocellulose (Bio-Rad), and immunoblotted with the above antibodies or mouse monoclonal anti-phosphotyrosine antibody (4G10, generously provided by Brian J. Druker, Oregon Health Sciences University). Bound antibodies were detected by chemiluminescence (NEN), followed by exposure to film. Stripping of blots was performed at 60°C for 30 min in stripping buffer, according to the manufacturer's protocol (Amersham Pharmacia, Piscataway, NJ).

**In vitro RAFTK kinase assay.** The immunoprecipitated complexes, obtained by immunoprecipitating lysates with anti-RAFTK antibodies, were washed three times with lysis buffer and twice in kinase buffer [20 mM Hepes (pH 7.4), 50 mM NaCl, 5 mM  $\text{MgCl}_2$ , 5 mM  $\text{MnCl}_2$ , 1 mM  $\text{Na}_2\text{VO}_4$ , and 20  $\mu$ M ATP]. The kinase assay was initiated by incubating the immune complex in kinase buffer containing 25  $\mu$ g of poly (Glu/Tyr, 4:1; 20-50 kDa; Sigma) and 5  $\mu$ Ci [ $\gamma$ - $^{32}$ P]-ATP at room temperature for 30 min. Reactions were terminated and analyzed as described (34,37).

**Migration assay.** Migration was performed as described (29). Results are expressed as the percentage of migrating cells as compared with the total number of cells (cells present in all Z-sections) and represent the means with SE.

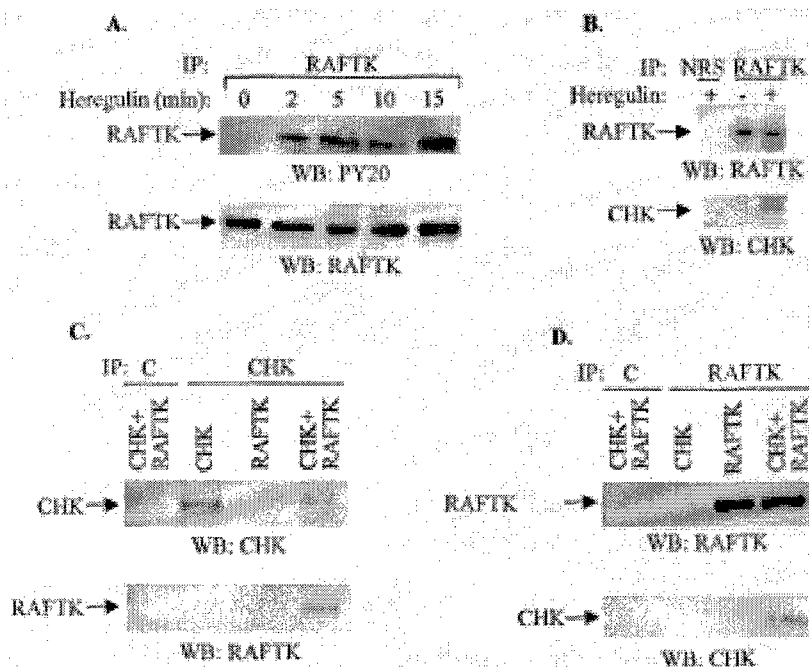


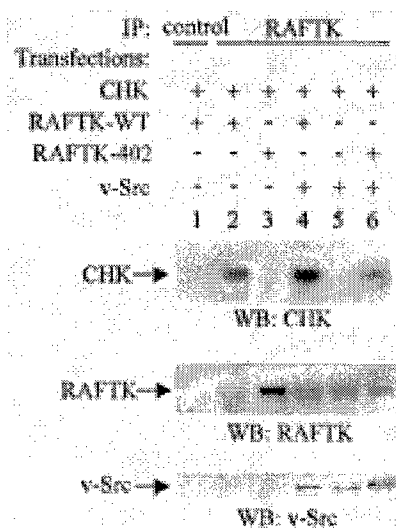
Figure 1. RAFTK association with CHK. (A), Tyrosine phosphorylation of RAFTK upon heregulin stimulation of T47D cells. Quiescent T47D cells were stimulated with 10 nM heregulin for different time periods. Cell lysates were immunoprecipitated with anti-RAFTK antibodies and separated on 7% SDS-PAGE, then blotted with PY20 anti-phosphotyrosine antibodies (upper panel). The same blot was stripped and blotted with anti-RAFTK antibodies (lower panel). (B), Association of RAFTK and CHK upon heregulin stimulation. T47D cells were transfected with pcDNA3neo-CHK. After 48 h, cells were stimulated with heregulin (10 nM for 15 min) and cell lysates were prepared and immunoprecipitated with either control rabbit serum (NRS) or RAFTK antibodies. Samples were separated on 7% SDS-PAGE and blotted with either RAFTK antibodies or CHK antibodies. (C and D), Co-immunoprecipitation of wild-type CHK and RAFTK in 293 cells. 293 cells were transiently transfected with plasmids containing full-length cDNAs encoding wild-type CHK (Flag-CHK) and RAFTK (Flag-RAFTK). Forty-eight hours post-transfection, cells were harvested and immunoprecipitated with antibody specific for CHK (C), or for RAFTK (D). Samples were separated on 10% SDS-PAGE and blotted with anti-CHK antibodies (C, upper panel) or anti-RAFTK antibodies (D, upper panel). Subsequently, the blots were stripped and reprobed with RAFTK (C, lower panel) and CHK (D, lower panel) antibodies, respectively. C, control antibody; CHK, FLAG-CHK; RAFTK, FLAG-RAFTK.

## Results

**Association of RAFTK with CHK in breast cancer cells.** Stimulation of T47D breast cancer cells with heregulin resulted in an increase in RAFTK tyrosine phosphorylation (Fig. 1A), which remains in accordance with our recently published results (13). Enhanced tyrosine phosphorylation of RAFTK is relatively specific for heregulin stimulation, as activation by EGF did not lead to increased RAFTK tyrosine phosphorylation (data not shown). To examine CHK interaction with RAFTK in breast cancer cells, T47D cells were transfected with the Flag-CHK or pcDNA3 vector alone. Transfected cells were stimulated with heregulin and co-immunoprecipitation studies were then performed. Anti-RAFTK antibodies co-immunoprecipitated CHK from T47D cells upon heregulin stimulation, while control antibody did not co-immunoprecipitate CHK (Fig. 1B). Furthermore, in a reciprocal experiment, anti-CHK antibodies co-immunoprecipitated RAFTK from T47D cells upon heregulin stimulation (data not shown). Thus, these data suggest that CHK associates with RAFTK in breast cancer cells upon heregulin stimulation.

**RAFTK and CHK specifically co-immunoprecipitate when overexpressed in 293 cells.** To elucidate further the interaction

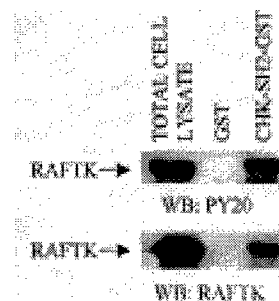
of RAFTK and CHK, 293 cells were transfected with either the pcDNA3 vector alone, Flag-CHK, or Flag-RAFTK construct. Forty-eight hours following transfection, cells were harvested and 1 mg of lysate was immunoprecipitated with either control antibodies, CHK-specific rabbit polyclonal antibodies, or RAFTK-specific goat polyclonal antibodies. Immunoprecipitates were separated by 7% SDS-PAGE, transferred to nitrocellulose, and immunoblotted, as described in Materials and methods. Immunoprecipitates obtained with the CHK antibody were probed with the RAFTK antibody, which revealed that RAFTK co-immunoprecipitated with the co-transfected CHK (Fig. 1C). The reciprocal experiment, in which immunoprecipitates obtained with the RAFTK antibody were probed with the CHK antibody, revealed that CHK was present in the RAFTK-specific immunocomplexes from cells transfected with both the CHK and RAFTK cDNAs (Fig. 1D). It should be noted that CHK alone was not immunoprecipitated by the RAFTK antibody, nor was RAFTK alone immunoprecipitated by the CHK antibody. Given that immunocomplexes with either antibody were obtained only from lysates of cells transfected with both the Flag-CHK and the Flag-RAFTK cDNAs, and that control experiments using cells transfected with CHK and immunoprecipitated with RAFTK-specific antibodies or the reciprocal RAFTK cDNA transfection followed by CHK-specific immunoprecipitation



**Figure 2.** Maximal interaction of CHK and RAFTK requires tyrosine 402, the Src-binding site of RAFTK. T47D cells were transiently transfected with plasmids containing the full-length cDNAs encoding wild-type CHK (Flag-CHK), wild-type RAFTK (Flag-RAFTK-WT) or the Src-binding site mutant Y402F (Flag-RAFTK-402) and the constitutively active oncogenic form of Src (v-Src). Forty-eight hours post-transfection, cells were lysed and 1 mg of lysate per sample was immunoprecipitated with RAFTK-specific goat polyclonal antibody or control antibodies. Products were separated by 7% SDS-PAGE, transferred to nitrocellulose and immunoblotted with CHK-specific rabbit polyclonal antibody. Subsequently, blots were stripped and reprobed with RAFTK antibody to confirm equivalent precipitation levels for the wild-type and mutant forms of RAFTK. In addition, aliquots of total cell lysates were analyzed for v-Src expression by Western blot analysis.

were negative, a specific interaction between the CHK and RAFTK proteins was indicated.

**Maximal interaction of CHK and RAFTK requires tyrosine 402, the Src-binding site of RAFTK.** In order to investigate the potential role of Src kinase in the aggregation of CHK with RAFTK, we transiently transfected T47D cells with various combinations of plasmids containing the coding sequences for wild-type CHK (Flag-CHK), wild-type RAFTK (Flag-RAFTK), Src-binding site mutant RAFTK (Flag-RAFTK-Y402F) and the constitutively active oncogenic Src (v-Src). Forty-eight hours following transfection, cells were lysed and 1 mg of lysate was precipitated with the goat polyclonal RAFTK-specific antibody or the control goat polyclonal anti-GST antibody. Following immunoprecipitation, precipitation products were separated on 7% SDS-PAGE, transferred to nitrocellulose, then immunoblotted with rabbit polyclonal CHK-specific antibody. The constitutively active, oncogenic form of Src (v-Src) enhanced the complex formation of wild-type CHK and wild-type RAFTK (Fig. 2, lane 4). No complex formation of wild-type CHK and Src-binding site mutant RAFTK (Flag-RAFTK-Y402F) was observed (Fig. 2, lane 3). Thus, the interaction of CHK with RAFTK seems to be mediated through the Src-binding site tyrosine residue 402 of RAFTK. Interestingly, v-Src partly restored the complex formation of CHK and the Src-binding mutant RAFTK (Fig. 2, lane 6).



**Figure 3.** Interaction between RAFTK and CHK is mediated by the SH2 domain of CHK. 293 cells were transiently transfected with RAFTK expression vector. Forty-eight hours post-transfection, cells were lysed and 500 µg of lysate per sample was precipitated with CHK-SH2-GST fusion proteins or GST (control) bound to glutathione-Sepharose beads. Products were separated by 7% SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-phosphotyrosine (PY20), then stripped and reprobed with anti-RAFTK antibody.

**Interaction between RAFTK and CHK is mediated by the SH2 domain of CHK.** Since the tyrosine residue 402 of RAFTK is important for aggregation of RAFTK and CHK, we decided to assess whether the SH2 domain of CHK is capable of binding to RAFTK. For this purpose, we used GST pull-down assay with the use of CHK-SH2-GST fusion protein (37). 293 cells were transiently transfected with wild-type RAFTK (Flag-RAFTK) construct and 48 h following transfection cells were lysed. One mg of total cell lysate was divided into two equal parts. One part was incubated for 16 h in 4°C with CHK-SH2-GST glutathione-Sepharose beads, while the other part was incubated with GST beads as a control. Precipitates were washed three times with the lysis buffer and the proteins were separated by 7% SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with antibodies against phosphotyrosine (PY20), then stripped and reprobed with antibodies against RAFTK. As shown in Fig. 3, CHK-SH2-GST strongly precipitated RAFTK from the lysate, while no precipitation was detected with GST alone. A similar pattern of precipitation was seen when we used CHK-SH2-SH3-GST fusion protein, while no precipitation of RAFTK by the CHK-SH3-GST fusion protein was seen (data not shown).

**RAFTK phosphorylation downstream of the activated growth factor receptor Neu is greatly reduced in the presence of CHK.** 293 cells were transiently transfected with cDNAs encoding wild-type RAFTK and activated Neu in the presence or absence of the cDNA encoding CHK. Forty-eight hours following transfection, cells were lysed and immunoprecipitated with RAFTK-specific goat polyclonal antibodies or control antibody. Products were separated by 7% SDS-PAGE, transferred to nitrocellulose, and immuno-blotted with antibodies against phosphotyrosine (4G10). Following immunoblotting with 4G10, blots were stripped and reprobed with the RAFTK antibody to confirm immunoprecipitation efficiency. Tyrosine phosphorylation and activation of wild-type RAFTK were increased in the presence of the Neu receptor (Fig. 4). Co-transfection of CHK with RAFTK and the activated Neu receptor resulted in a significant reduction of RAFTK tyrosine phosphorylation and activation (Fig. 4). In

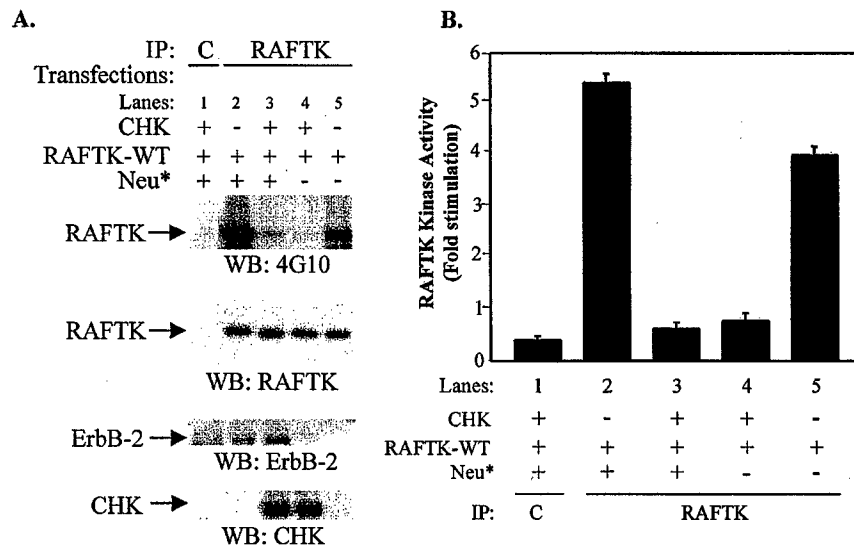


Figure 4. Co-transfection of CHK reduces the tyrosine phosphorylation of RAFTK. (A), 293 cells were transiently transfected with plasmids containing the cDNAs for wild-type RAFTK (WT), CHK and the activated neu receptor (Neu\*). Forty-eight hours post-transfection, cells were lysed and 1 mg of lysate per sample was immunoprecipitated with RAFTK-specific antibody or control antibody (C). Products were separated by 7% SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-phosphotyrosine (4G10). Subsequently, the blots were stripped and reprobed with the RAFTK antibody to confirm equivalent immunoprecipitation levels for all lanes. In addition, aliquots of the total cell lysates were hybridized with ErbB-2 and CHK antibodies. (B), Kinase activity of RAFTK. Total cell lysates of transfected 293 cells (as described in A) were prepared, immunoprecipitated with anti-RAFTK antibody (lanes 2-5) or with control antibody (lane 1) and then subjected to an *in vitro* kinase assay, as described in Materials and methods. C, control.

the presence of CHK without transfected activated Neu, levels of RAFTK tyrosine phosphorylation were at merely detectable levels (Fig. 4A, lane 4). Stripping and reblotting with RAFTK antibody revealed equivalent immunoprecipitation efficiency (Fig. 4A). These data indicate that the phosphorylation state of RAFTK can be negatively regulated by CHK, probably through the down-regulation of Src tyrosine kinases activated downstream of growth factor receptor activation.

CHK utilizes at least two different mechanisms to down-regulate RAFTK. To assess whether kinase activity of CHK is crucial for down-regulation of RAFTK phosphorylation, we used the kinase inactive mutant of CHK (dead-kinase CHK, dkCHK) (41). 293 cells were transfected with either the pcDNA3 vector alone, Flag-CHK, Flag-dkCHK, and/or Flag-RAFTK construct. Forty-eight hours following transfection, cells were harvested and 1 mg of lysate was immunoprecipitated with RAFTK-specific antibodies. Immunoprecipitates were separated by 7% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-phosphotyrosine PY20 antibodies. Then, the membrane was stripped and reprobed with RAFTK antibody (Fig. 5, bottom panel). Expression of CHK proteins was assessed in total cell lysates by Western blot procedure (Fig. 5, top panel). As shown in Fig. 5 (middle panel), in cells transfected both with RAFTK alone or RAFTK + pcDNA3 vector, marked phosphorylation of RAFTK was detected. Co-expression of wild-type CHK along with RAFTK strongly reduced the level of tyrosine phosphorylation of RAFTK. However, a significant decrease in RAFTK phosphorylation was also seen in the cells co-transfected with dkCHK and RAFTK, suggesting that the presence of CHK protein without kinase activity is at least partly sufficient to negatively regulate RAFTK phosphorylation.

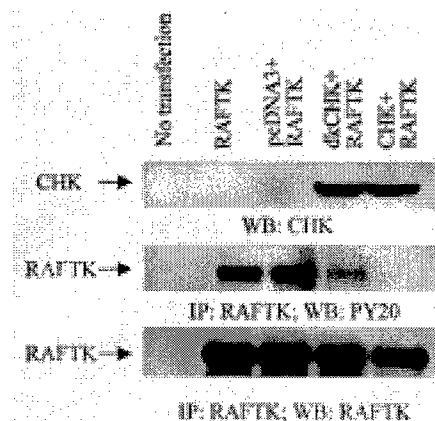


Figure 5. Effects of wild-type or dead-kinase (dk)CHK on RAFTK phosphorylation in 293 cells. 293 cells were transiently transfected with plasmids containing the cDNAs for CHK, dkCHK, and/or wild-type RAFTK. Forty-eight hours post-transfection, cells were lysed and 1 mg of lysate per sample was immunoprecipitated with RAFTK antibody. Products were separated by 7% SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-phosphotyrosine (PY20) (middle panel), then stripped and reprobed with anti-RAFTK antibody (bottom panel) to confirm equivalent immunoprecipitation efficiency for all samples. Expression of CHK proteins was assessed by Western blot analysis with anti-CHK antibody (upper panel).

**CHK negatively regulates paxillin phosphorylation.** We have shown that paxillin is an *in vivo* binding partner and substrate for the tyrosine kinase activity of RAFTK in hematopoietic cells. In order to further evaluate the functional relevance of the interaction between CHK and RAFTK, we analyzed the phosphorylation state of paxillin in the presence of CHK, c-Src, and/or the wild-type RAFTK in comparison to paxillin

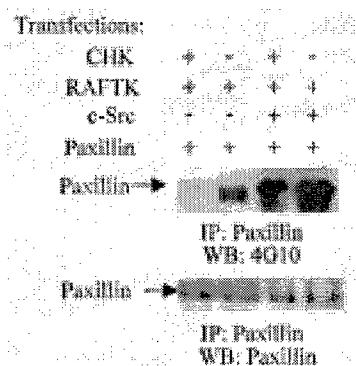


Figure 6. CHK decreases the phosphorylation of paxillin. 293 cells were transiently transfected with plasmids containing the cDNAs for CHK, c-Src, paxillin, and/or the wild-type RAFTK. Forty-eight hours post-transfection, cells were lysed and 1 mg of lysate per sample was immunoprecipitated with paxillin antibody. Products were separated by 7% SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-phosphotyrosine (4G10). Subsequently, the blots were stripped and reprobed with paxillin antibody to confirm equivalent immunoprecipitation efficiency for all samples.

phosphorylation in the absence of CHK in transiently transfected 293 cells (Fig. 6). Tyrosine phosphorylation levels of paxillin when co-transfected with CHK and wild-type RAFTK were decreased over those obtained upon transfection of paxillin and wild-type RAFTK alone (Fig. 6, top panel, lanes 1 and 2). Further enhancement of phosphorylation was observed when c-Src was co-transfected with RAFTK-WT and paxillin. However, in the presence of CHK, the level of paxillin phosphorylation was decreased (Fig. 6, top panel, lanes 4 and 3, respectively).

**Overexpression of CHK inhibited the migration of breast cancer cells in response to HRG.** We have shown recently that expression of RAFTK enhances HRG-stimulated breast cancer cell invasion (13). To elucidate the effects of CHK on the HRG-induced cell migration of RAFTK-expressing breast cancer cells, inducible CHK stably transfected T47D cells were generated and characterized (Fig. 7A). Inducible stably transfected T47D cell clones, in the presence of tetracycline (expressing endogenous CHK) or in the absence of tetracycline (thus overexpressing CHK), as well as control T47D cells, were treated with HRG (10 nM) for 16 h. Cell migration was then analyzed using Boyden Chambers. The migration of T47D cells overexpressing CHK (-Tet) was significantly inhibited in the presence of HRG (Fig. 7B and C), as compared to the control T47D cells alone (without CHK) (Fig. 7B) or cells with the endogenously expressed CHK (+Tet) (Fig. 7B and C). These results demonstrate that CHK negatively regulates the HRG-induced migration of RAFTK-expressing breast cancer cells.

## Discussion

Abnormalities in the expression, structure, or activity of numerous proteins governing cell proliferation, survival, migration, and invasiveness contribute to the development and maintenance of the malignant phenotype. Well-known

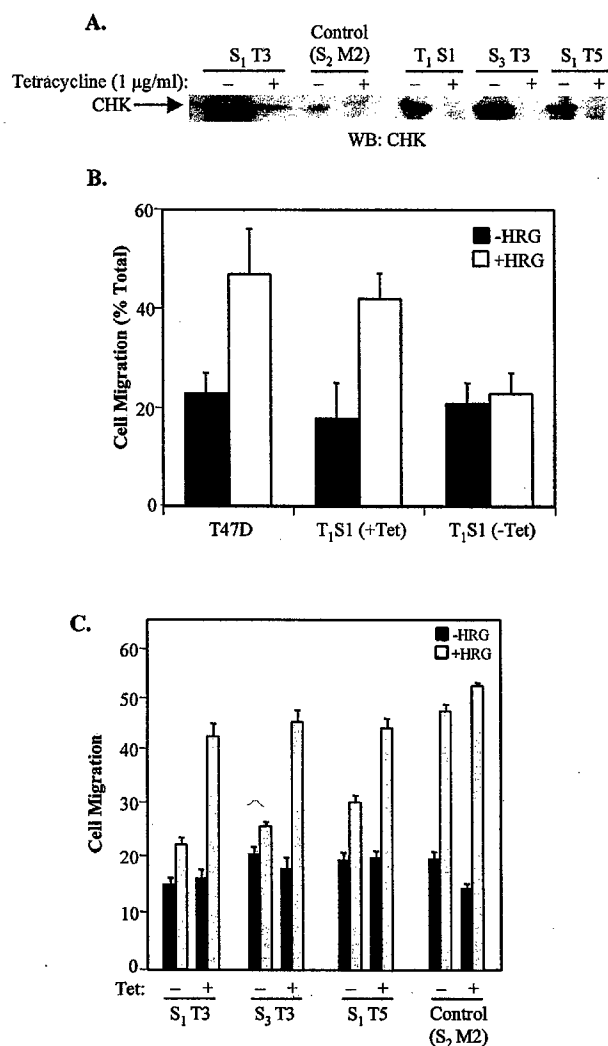


Figure 7. Inducible overexpression of CHK inhibited cell migration in T47D cells. (A), Western blot analysis of CHK protein expression in several selected clones of inducible stably transfected T47D cells expressing CHK, in the presence (+) or absence (-) of tetracycline (1 µg/ml) for 48 h. Forty µg of total cell lysates were prepared from these cell clones and examined by Western blot analysis using anti-CHK antibody. Control, T47D cells transfected with vector alone. (B and C), Effect of CHK on the migration of breast cancer cells: T47D cells or inducible stably transfected cells in the presence (+Tet) (1 µg/ml) (expressing endogenous CHK) or absence (-Tet) (overexpressing CHK) of tetracycline were untreated or treated with HRG (10 nM) for 16 h. Cell migration was then analyzed using Boyden Chambers. The data shown are mean values (± SEM) of three experiments done in triplicate. The number of migrating cells was quantitated as described in Materials and methods.

examples of such proteins can be found within the family of receptor tyrosine kinases and their ligands. HER2/Neu (also termed ErbB-2) is a transmembrane 185 kDa receptor with intrinsic tyrosine kinase activity. Amplification of the HER2 gene and overexpression of its product (which is detected in 10-40% of human breast tumors) induce malignant transformation of mammary cells. Recent studies suggest that HER2/Neu is a ligand orphan receptor that amplifies the signal provided by other receptors of the HER family after forming ligand-induced heterodimers with them. Ligand-dependent



activation of HER1, HER3, and HER4 by EGF or heregulin (HRG) results in HER2 activation (reviewed in ref. 42). In accordance with these observations, experiments with transgenic mice have proven heregulin to contribute to the development of mammary adenocarcinomas (43), and expression of HRG was identified to correlate with a more aggressive/invasive phenotype of breast cancer cells (44). Although HRG was reported to induce the formation of a motile actin cytoskeleton structure through PI-3 kinase and p21 activated kinase 1 (PAK1), the detailed mechanism of this HRG-induced cell migration remains unknown. Cell migration is a spatially and temporally coordinated process that is regulated by a combination of cellular responses: cell polarization, membrane extension at the leading edge of the cell, formation and turnover of cell adhesion complexes, and the contraction of the cell rear (45,46). Our recent studies showed that heregulin induced the formation of a multi-protein complex with RAFTK. The proteins participating in this complex include p190 RhoGAP, p120 RasGAP and ErbB-2 (13,29). In this complex, RAFTK played an essential role in mediating the tyrosine phosphorylation of p190 by Src, and thus in controlling the association of p190 and RasGAP. Since the small G-proteins are key mediators of cell migration, RAFTK may also play an important role in cell migration through these G-proteins. RAFTK has been reported to regulate actin-based cytoskeletal reorganization and cell adhesion in various cell types (1,4). It is conceivable that RAFTK might mediate HRG-induced cell migration through the regulation of actin-based cytoskeletal reorganization and/or cell adhesion. As activation of RAFTK remains in a close correlation with the activity of Src family kinases, we used a previously identified inhibitor of Src kinase activity, Csk homologous kinase (CHK), to oppose activation of RAFTK.

In the present study, we demonstrated that HRG induced the tyrosine phosphorylation of RAFTK and its association with CHK in breast cancer cells. The transfection studies reveal the association of CHK with RAFTK to be mediated via the Src-binding site, Tyr-402, on RAFTK, and the SH2 domain of CHK (Figs. 2 and 3, respectively). These data suggest that interaction between CHK and RAFTK is at least in part direct. However, as shown in Fig. 2, co-expression of v-Src enhanced association of CHK with wild-type RAFTK and partly restored co-immunoprecipitation of CHK by anti-RAFTK antibodies when RAFTK(Y402F) mutant was used. These results suggest also indirect interaction between RAFTK and CHK. Both RAFTK and CHK were demonstrated to bind a range of other molecules. Most probably, v-Src phosphorylates tyrosine moieties in proteins already associated with RAFTK (via other ways than binding to Tyr-402), which produces new docking sites for the CHK-SH2 domain. Therefore, our data demonstrate the possibility of both a direct and indirect association of CHK and RAFTK.

Our recently published data and the results presented in Fig. 1 demonstrate induction of RAFTK phosphorylation downstream to HER2/Neu activation. When overexpressed in 293 cells, wild-type RAFTK undergoes significant phosphorylation and activation even in the absence of Neu-mediated signaling (Figs. 3-5). These conditions are sufficient for CHK to associate with RAFTK. Nevertheless, as shown

in Fig. 4, co-transfection of RAFTK with the activated Neu receptor produces further enhancement of RAFTK phosphorylation and kinase activity, confirming participation of RAFTK in Neu-mediated signaling. Importantly, association of RAFTK with CHK results in a remarkable decrease in phosphorylation (Fig. 4A) and in the catalytic activity (Fig. 4B) of RAFTK, stimulated both by overexpression and/or co-expression of Neu\*.

To elucidate the role of the kinase activity of CHK in the negative regulation of RAFTK activation, we used a dead-kinase mutant of CHK. We observed, that while co-expression of dkCHK with RAFTK in 293 cells was able to markedly diminish the level of phosphorylation of RAFTK, this effect was further potentiated when wild-type CHK was used (Fig. 5). These results suggest the existence of at least two mechanisms of negative regulation of RAFTK activation by CHK. The first would be a competition reaction between CHK and Src on the 402 binding site of RAFTK, in which CHK may displace Src from Y402 of RAFTK. The second, would be the inhibition of Src-kinase activity by CHK by phosphorylation of a conserved carboxyl-terminal tyrosine (Tyr-527).

In the course of our studies on interactions between RAFTK-mediated signaling and CHK, we assessed the effects of CHK on phosphorylation of paxillin in 293 cells transfected with CHK, RAFTK, and/or c-Src DNAs. The phosphorylation of paxillin is known to be mediated by both RAFTK and c-Src. We observed a marked decrease in phosphorylation of paxillin when CHK was co-expressed either with RAFTK only or RAFTK + c-Src (Fig. 6). Thus, the effects of CHK on RAFTK phosphorylation and activation impair the downstream signaling from RAFTK.

In addition, we demonstrated that conditional overexpression of CHK inhibited the HRG-induced migration of RAFTK-expressing T47D breast cancer cells (Fig. 7). Our recent data suggest that expression of CHK markedly diminishes HRG-induced invasion of MCF-7 breast cancer cells (41), which do not express RAFTK (data not shown). Therefore, to establish the link between inhibition of the RAFTK signaling pathway by CHK and the effects of CHK on HRG-induced cell migration, further studies are necessary. We generated inducible stable transfected T47D breast cancer cells which express CHK and endogenous RAFTK. As shown in Fig. 7, CHK inhibited the migration of T47D breast cells in response to HRG.

Taken together, these results indicate for the first time that CHK interacts with and can regulate RAFTK, and reveal novel findings regarding the role of CHK as a potential mediator of cytoskeletal function as well as its involvement in the migration of breast cancer cells.

Based on previously published data (6) and our results, we have proposed a model for the molecular interaction of RAFTK, Src, and CHK (Fig. 8). HRG stimulation induces the activation of RAFTK, which leads to the autophosphorylation of the tyrosine 402. It has been shown previously that the tyrosine-phosphorylated 402 of RAFTK can bind to the SH2 domain of Src, resulting in the activation of Src (6). In addition, Src has been reported to phosphorylate the tyrosine 881 of RAFTK (47,48). Based on the conserved amino acid sequence motifs (49) and the results from the

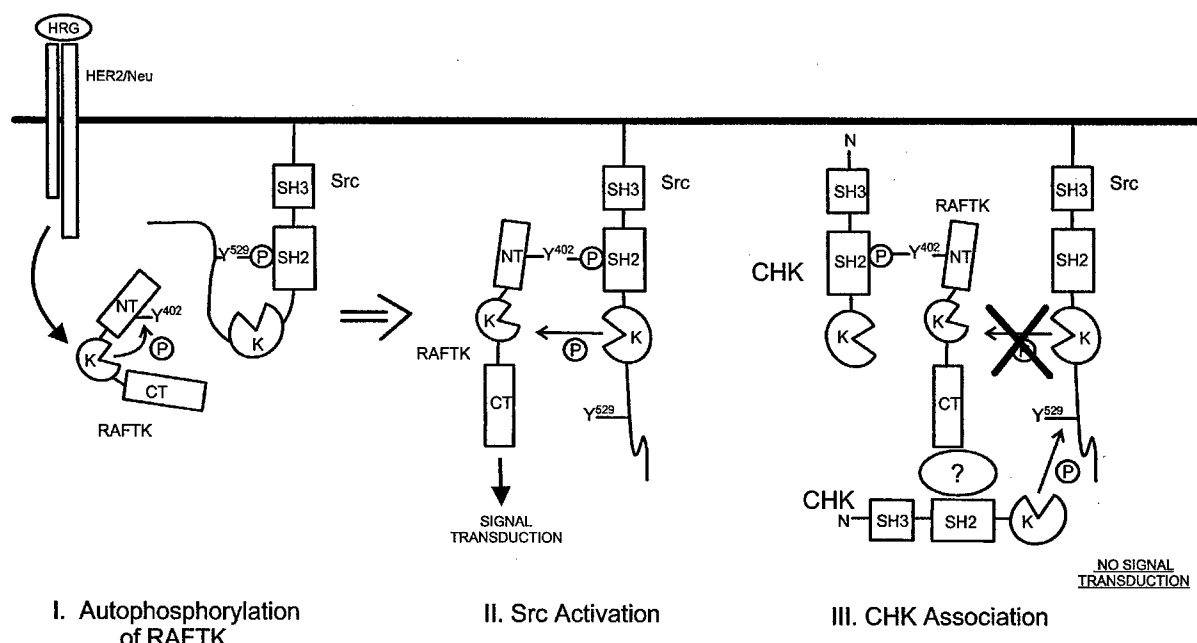


Figure 8. A proposed model for the molecular interaction of RAFTK, Src and CHK. (I), Upon activation, RAFTK autophosphorylates the tyrosine residue at 402, which is a Src-binding site. The autophosphorylated tyrosine at 402 of RAFTK binds to the SH2 domain of Src, which releases the negative regulatory binding of phospho-Tyr-529, resulting in Src activation. (II), Activated Src phosphorylates several sites of RAFTK including amino acid residues at 579, 580 and 881, which may lead to conformational changes and increases in RAFTK activity. (III), CHK associates with the activated RAFTK and inhibits its activation either by competing with Src to bind Tyr-402 or by negative regulation of Src kinase activity (CHK phosphorylates the tyrosine residues at 529 of Src, leading to the inactivation of Src). CT, C-terminal domain; K, kinase domain; NT, N-terminal domain; SH2, Src homology 2 domain; SH3, Src homology 3 domain.

study of the homologous kinase FAK (50), Src can potentially phosphorylate the tyrosine 579 and 580 of the RAFTK kinase domain. These events lead to activation of RAFTK. Our results demonstrate that co-expression of CHK leads to the negative regulation of this process by more than one mechanism.

In summary, this report demonstrates the *in vivo* association of the cytoplasmic tyrosine kinase CHK with the cytoplasmic focal adhesion-related tyrosine kinase RAFTK. This interaction results in the reduction of RAFTK tyrosine phosphorylation and catalytic activity. Furthermore, inducible overexpression of CHK down-regulated the HRG-induced migration of RAFTK-expressing cells. These findings indicate the role of CHK or its derivatives as very promising tools to target Neu-Src-RAFTK-mediated intracellular signaling in breast cancer cells.

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## Overexpression of the Csk homologous kinase facilitates phosphorylation of Akt/PKB in MCF-7 cells

RADOSLAW ZAGOZDZON, CÉCILE BOUGERET, YIGONG FU and HAVA KARSENTY AVRAHAM

Division of Experimental Medicine, Beth Israel Deaconess Medical Center,  
Harvard Medical School, 4 Blackfan Circle, Boston, MA 02115, USA

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**Abstract.** The serine/threonine kinase Akt has recently been the focus of intense research. Akt activation requires the phosphorylation of both Thr-308 and Ser-473. Src kinase was shown to induce activation of Akt, while Lyn kinase seems to inhibit this activation. In the present study, we investigated the effect of overexpressing the Csk homologous kinase (CHK), an inhibitor of Src-family kinases, on the phosphorylation of Akt induced by two different factors: heregulin or cisplatin. We used MCF-7 cells stably overexpressing the wild-type CHK [CHK(wt)] or dead-kinase CHK [CHK(dk)]. We observed that in MCF-7 CHK(wt) cells Lyn kinase activity was more profoundly inhibited than Src kinase activity. When the cells were stimulated with heregulin or cisplatin, Akt phosphorylation occurred more rapidly in MCF-7 CHK(wt) cells in comparison to the other clones used. Interestingly, MCF-7 CHK(wt) cells *in vitro* were markedly more resistant to cisplatin than the other clones used in the experiments, and surprisingly chemical inhibition of Akt phosphorylation did not influence this resistance. In summary, our results show facilitation of Akt phosphorylation by the overexpression of CHK, and provide new insight into the putative role of CHK in human cancer.

### Introduction

The serine/threonine kinase Akt, or protein kinase B (PKB), consists of a family of highly conserved serine/threonine kinases including Akt1 and Akt2. It was originally identified as a proto-oncogene from rodent T cells (1). This protein has

recently been a focus of intense research, as it appears to act as a transducer of signals initiated by numerous growth factor receptors as well as to lie in the crossroads of multiple cellular signaling pathways induced by other factors, e.g. DNA damaging agents (reviewed in ref. 2). It is known that one of the major activities of Akt is to mediate cell survival (3), which, together with the recent discovery of the tumor suppressor PTEN as an inhibitor of Akt kinase activity (4), suggest that Akt is a critical factor in the genesis of cancer. Thus, elucidation of the mechanisms of Akt regulation as well as the physiological functions of Akt seems to be important for the understanding of cellular metabolism, apoptosis, and cancer.

To become activated, usually in a PI3-K-dependent manner, Akt requires the phosphorylation of both Thr-308 and Ser-473 (5). Recent studies show that the activity of Akt could be differentially regulated by Src-family kinases. Specifically, Akt was shown to be activated by active Src kinase (6), while Lyn kinase was demonstrated to negatively influence the function of Akt (7,8). Therefore, it can be assumed that factors modifying the activity of Src-family kinases may affect Akt activation as well.

One of the most known inhibitors of Src-family kinases is the ubiquitously expressed Csk (C-terminal Src kinase) (9). Previously, we and others have identified a cytoplasmic tyrosine kinase CHK (Csk homologous kinase) that shares about 50% homology with Csk (10). It contains SH2, SH3 and tyrosine kinase domains and is able to phosphorylate the inhibitory C-terminal tyrosine of Src (11) and of several Src-related enzymes, including Lck (12), Fyn (13) and Lyn (11,14,15). Unlike Csk, CHK is primarily expressed solely in hematopoietic cells and in brain. Nevertheless, our studies revealed (by RT-PCR and immunohistochemistry) that the CHK protein is expressed in 70 out of 80 breast carcinoma specimens, but not in normal or benign (fibroadenoma) breast tissues (0/19) (16). Although CHK expression in primary breast tumors is very low compared to that of Csk, as it could not be detected by Western blotting and/or immunoprecipitation, the role of CHK in the development of breast cancer cannot be excluded. In our most recent studies, we have shown that stable overexpression of wild-type CHK [CHK(wt)] in MCF-7 breast cancer cells suppressed their malignant growth and was related to the inhibition of Src and Lyn protein expression and kinase activity (16). However, expression of dead-kinase mutant [CHK(dk)] did not exert that effect (16). Of note, Lyn kinase activity was more

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**Correspondence to:** Dr Hava Karsenty Avraham, Division of Experimental Medicine, Beth Israel Deaconess Medical Center, Harvard Institutes of Medicine, 3rd floor, 4 Blackfan Circle, Boston, MA 02115, USA  
E-mail: havraham@caregroup.harvard.edu

**Abbreviations:** CHK, Csk homologous kinase; Csk, C-terminal Src kinase; PI3-K, phosphatidylinositol 3-kinase; PKB, protein kinase B; XIAP, X-linked inhibitor of apoptosis protein

**Key words:** breast cancer, chemotherapy, growth factor, growth factor receptor, signal transduction

profoundly inhibited than Src kinase activity. Similarly, it was demonstrated that Csk also inactivates Lyn with a significantly higher efficiency than Src (17). In this study, we decided to assess the effect of overexpressing CHK in MCF-7 breast cancer cells on the phosphorylation of Akt induced upon stimulation with either heregulin, as a representative growth factor, or cisplatin, as one of the known DNA damaging agents. Both factors were previously shown to activate Akt (18,19). Additionally, Akt phosphorylation was recently suggested to be one of the most important factors in the resistance to cisplatin, achieved by overexpressing the X-linked inhibitor of apoptosis protein (XIAP) (20). Therefore, survival of the MCF-7 cells following the cisplatin treatment in our model was also assessed.

## Materials and methods

**Cell line and reagents.** MCF-7 cells were obtained from ATCC (American Type Culture Collection, Rockville, MD). Cells were maintained in the culture medium: RPMI 1640 medium (Cellgro, Mediatech Inc., Herndon, VA) supplemented with 10% FBS (Sigma, St. Louis, MO), antibiotics, L-glutamine (2 mM), and sodium pyruvate (1 mM) (all from Cellgro). The generation of the kinase-inactivating lysine (AAG) to arginine (GCG) mutation in the CHK gene, the construction of CHK(wt)- and CHK(dk)-encoding pcDNA3 plasmids as well as stable transfections with pcDNA3neo or pcDNA3-CHK-encoding plasmids of MCF-7 cells were described in detail previously (16,21). Recombinant human heregulin ( $\gamma$ HRG- $\beta$ 1, 177-244) was generously provided by Dr Mark X. Sliwkowski (Genentech Inc., San Francisco, CA). Cisplatin was purchased from American Pharmaceutical Partners, Inc. (Los Angeles, CA). LY294002, PD98059, and Rapamycin were purchased from Cell Signaling Technology (Beverly, MA).

**Western blot analysis of protein expression.** For Western blot analyses, cells were scraped off the plates in cell lysis buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 10% glycerol) containing anti-phosphatase (1 mM sodium orthovanadate) and anti-proteases (Complete<sup>TM</sup> - Protease Inhibitor Cocktail Tablets (Boehringer Mannheim GmbH, Mannheim, Germany) and lysed for 45 min at 4°C. Protein concentrations were determined using a commercial protein assay (Bio-Rad, Hercules, CA). Total protein extracts (60  $\mu$ g) were electrophoretically separated on 10% polyacrylamide-SDS gels, transferred to PVDF membrane, and probed with antibodies against CHK (Lsk, Santa Cruz Biotechnology, Santa Cruz, CA), Akt, and phospho-Akt (Ser-473) (Cell Signaling Technology). Immunodetection was performed using the enhanced chemiluminescence system (NEN, Life Science Products, Inc., Boston, MA). Subsequently, blot membranes were stripped for 40 min at 60°C in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) and reprobed for actin (Chemicon International, Inc., Temecula, CA).

**In vitro tyrosine kinase assay.** MCF-7 cells were grown on 100-mm Petri dishes until 80% confluent and then total protein

extracts were prepared as described above. Subsequently, 1 mg of protein was immunoprecipitated using antibodies against CHK (Lsk, Santa Cruz Biotechnology), Src (clone GD11, Upstate Biotechnology) or Lyn (Santa Cruz Biotechnology). The immunoprecipitates were washed 3 times with the lysis buffer and then resuspended in 30  $\mu$ l kinase buffer (50 mM Tris-HCl, pH 7.4, 10 mM MnCl<sub>2</sub>, 10 mM MgCl<sub>2</sub>, 0.1% Triton X-100, 1 mM dithiothreitol) containing phosphatase- and protease-inhibitors (see above), 0.25 mg/ml poly(Glu/Tyr)4:1 (Sigma) as an exogenous kinase substrate, 10  $\mu$ M unlabeled ATP and 10  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP (6000 Ci/mmol, NEN). After 10 min at 30°C, the reaction was stopped by adding SDS-sample buffer, followed by boiling the samples for 10 min. Subsequently, the samples were resolved on 12% polyacrylamide-SDS gels and the gels were stained with Coomassie blue. The labeled poly(Glu/Tyr) was excised from the gel and the radioactivity was counted.

**Cell survival assay.** To assess the sensitivity of MCF-7 cells to cisplatin, a standard MTT assay was used. Briefly, tumor cells were dispensed in a 96-well flat-bottomed microtiter plate at a concentration of  $1 \times 10^5$  cells in 200  $\mu$ l. Twenty-four hours after seeding, serial dilutions of cisplatin in 50  $\mu$ l of culture medium were added to the cell cultures. In the experiment using the signal transduction pathway inhibitors, PI3-kinase (LY294002), MEK1 (PD98059), or FRAP/mTOR (Rapamycin) inhibitors were added to the cell cultures at specified concentrations 1 h prior to cisplatin treatment. After 24-h incubation, 30  $\mu$ l of 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT, Sigma) solution (3 mg/ml) was added into each well. Plates were centrifuged 4 h later (200 g/10 min), and the supernatants were carefully removed and replaced with 200  $\mu$ l of 2-propanol. After a 15-min incubation at room temperature with shaking (200 rpm), plates were read using a standard ELISA reader with 490 nm/650 nm filters. The survival of the cells was expressed as the optical density (OD 490-650) or as the relative viability (% of control cell cultures incubated with culture medium only). In the studies on the sensitivity of MCF-7 cells to a range of cisplatin concentrations, the results of the three independent experiments performed were concordant with each other. Thus, the data were pooled and expressed as a single graph. The differences in data were calculated by using the Student's t-test. P values <0.05 were considered to be statistically significant.

## Results

**Inhibition of Src and Lyn activities by overexpression of CHK in MCF-7 cells.** In this set of experiments, we assessed the modulation of Src and Lyn kinase activities in cells expressing CHK dead-kinase (dk) or wild-type (wt) (Fig. 1A). As shown in Fig. 1B, both Lyn and Src activities were markedly lower in cells expressing CHK(wt) in comparison with all other clones. This inhibition correlated with the kinase activity of CHK (Fig. 1C). Of note, the activity of Lyn was impaired to a greater extent (60.2% inhibition vs. the parental cell line) than that of Src (38.7% inhibition). These results are concordant with our prior observations (16). Of note, the expression level of Lyn, as assessed by Western

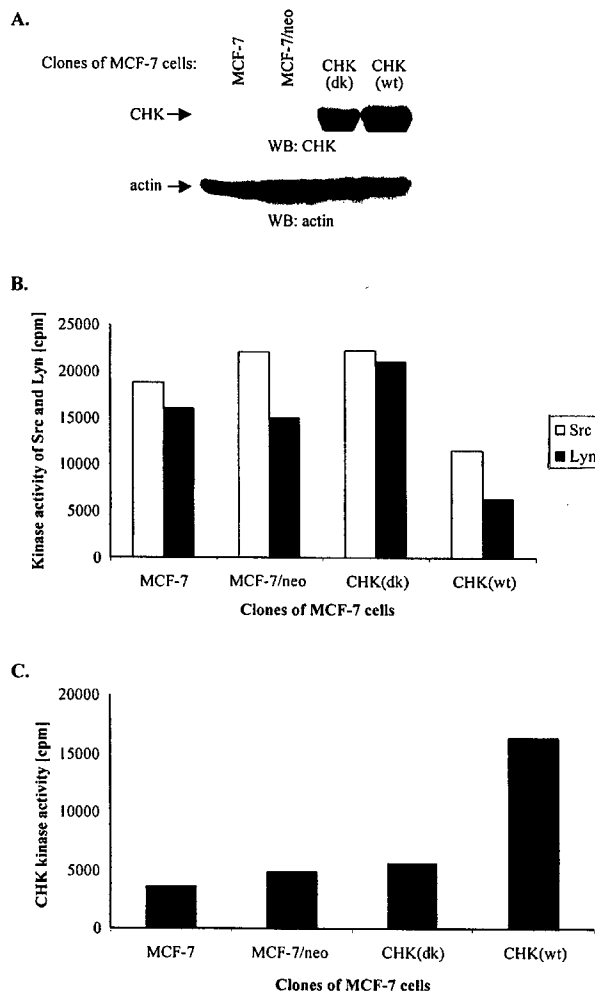


Figure 1. Characterization of the expression of CHK proteins (A), and of the activities of Src, Lyn (B), and CHK (C) tyrosine kinases in MCF-7 cells. (A), Total protein lysates were prepared from MCF-7 cells; parental (MCF-7), transfected with empty vector (MCF-7/neo), and expressing CHK (wt) or (dk). 60  $\mu$ g of total protein lysates were separated on 10% SDS-PAGE and immunoblotted with Lsk antibody, then stripped and reprobed for actin as described in Materials and methods. (B and C), MCF-7 cells; parental (MCF-7), transfected with empty vector (MCF-7/neo), and expressing CHK (wt) or (dk), were seeded onto 100-mm Petri dishes and grown until 80% confluence. Thereafter, cells were starved overnight in 1% FBS followed by 4-h starvation in serum-free culture medium. The cells were then washed twice with ice-cold PBS, and total protein lysates were prepared. One mg of total lysate was immunoprecipitated with the appropriate antibodies, and an *in vitro* kinase assay using poly(Glu/Tyr) as an exogenous substrate was performed (see Materials and methods for details).

blotting, was also more deeply suppressed than the expression level of Src in cells overexpressing CHK(wt) in comparison with the other clones studied (data not shown).

**Kinetics of the heregulin-induced phosphorylation of Akt.** MCF-7 cells, parental (MCF-7) or expressing CHK(wt), were stimulated with heregulin. Western blot analysis of the phosphorylation of Akt on Ser-473 was then performed. As shown in Fig. 2, a low level of Akt phosphorylation was already detected in MCF-7 CHK(wt) cells after 1-min

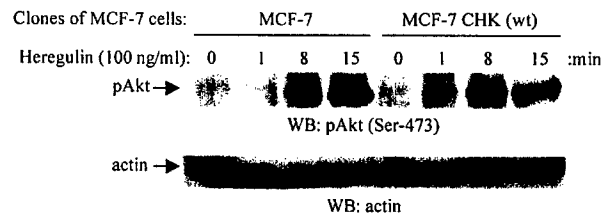


Figure 2. Influence of incubation with heregulin on Akt phosphorylation on Ser-473 in MCF-7 cells. MCF-7 cells; parental (MCF-7) or expressing CHK (wt), were seeded onto 100-mm Petri dishes and grown until 80% confluence. Thereafter, cells were starved overnight in 1% FBS followed by 4-h starvation in serum-free culture medium. After this time period, heregulin was added to the culture medium at a final concentration of 100 ng/ml. Cells were incubated in the presence of heregulin for 1, 8 or 15 min. Cells were washed twice with ice-cold PBS and total protein lysates were prepared as described in Materials and methods. Sixty  $\mu$ g of total lysate was separated on 10% SDS-PAGE and immunoblotted with phospho-Akt (pAkt) antibody, then stripped and reprobed for actin as described in Materials and methods.

incubation, while no phosphorylation of Akt could be seen in the parental cells at the same time point. Similarly, after 8 min, the phosphorylation of Akt was again more pronounced in the cells expressing CHK(wt).

**Cisplatin-induced phosphorylation of Akt.** As cisplatin is known to induce Akt activation, the effects of this drug in our model were studied. Four clones of MCF-7 cells: parental (MCF-7), transfected with empty vector (neo), and expressing CHK(dk) or (wt), were stimulated with cisplatin. Next, Western blot analysis of the phosphorylation of Akt on Ser-473 was performed. Additionally, cell lysates from non-treated cells were used for Western blotting with anti-Akt antibody, and no noticeable differences were observed in terms of Akt expression between the four clones of MCF-7 cells (Fig. 3A). As shown in Fig. 3B, no phosphorylation of Akt was detected in the non-treated cells. However, in MCF-7 cells expressing CHK(wt), marked phosphorylation of Akt was already detected after 1-h incubation, while only minor changes were observed in the other clones of MCF-7. Clearly visible changes in Akt phosphorylation were noted in all cell types following 4-h incubation. Nevertheless, in cells expressing CHK(wt), this phenomenon seemed to be stronger than in the other clones. Noteworthy, in cells expressing CHK(dk), the phosphorylation of Akt appeared to be even weaker than in the parental or neo MCF-7 cells.

**Survival of MCF-7 cells after treatment with cisplatin and influence of signal transduction pathway inhibitors on the sensitivity of MCF-7 cells to cisplatin.** To investigate the sensitivity of MCF-7 cells to cisplatin *in vitro*, different clones of the MCF-7 cells were treated with cisplatin, and cell survival was measured. As shown in Fig. 4A, the clone expressing CHK(wt) was markedly more resistant to cisplatin treatment as compared to all other clones in the experiment. Similar results were obtained with the second clone of MCF-7 cells expressing CHK(wt) [for characterization of both clones see (16)] (data not shown). Of note, the clone expressing CHK(dk) seemed to be more sensitive to cisplatin

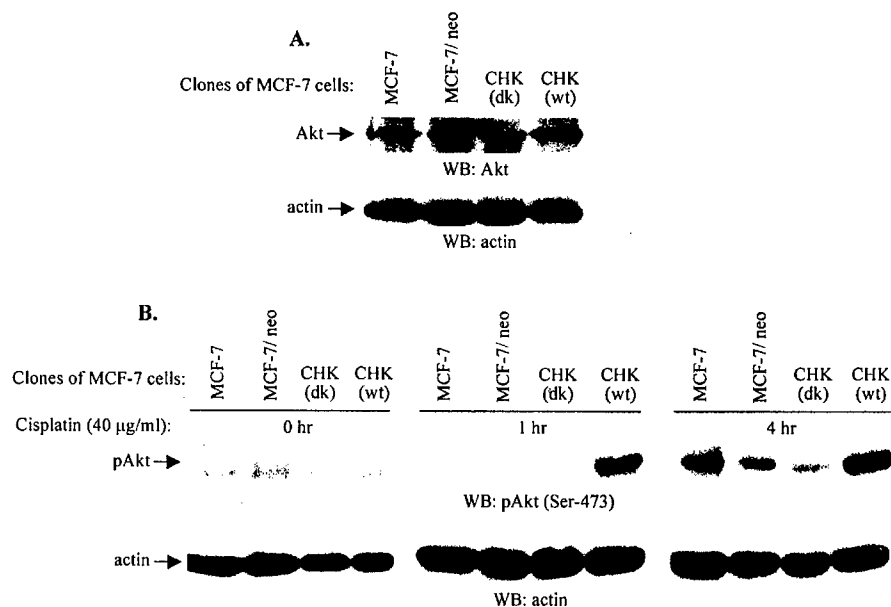


Figure 3. Expression of Akt protein in quiescent MCF-7 cells (A) and the influence of incubation with cisplatin on the phosphorylation of Akt on Ser-473 in MCF-7 cells (B). MCF-7 cells; parental (MCF-7), transfected with empty vector (MCF-7/neo), and expressing CHK (wt) or (dk), were seeded onto 100-mm Petri dishes and grown until 80% confluence. Subsequently, cells were starved overnight in 1% FBS followed by 4-h starvation in serum-free culture medium. After this period, cisplatin was added to the culture medium to a final concentration of 40 µg/ml. Cells were incubated in the presence of cisplatin for 1 or 4 h. Thereafter, cells were washed twice with ice-cold PBS and total protein lysates were prepared as described in Materials and methods. Sixty µg of total lysate was separated on 10% SDS-PAGE and immunoblotted with Akt (A) or phospho-Akt (pAkt) antibodies (B), then stripped and reprobed for actin as described in Materials and methods.

than the parental or neo MCF-7 cells. Similar differences were also observed when the clones of MCF-7 cells were incubated with cisplatin for 48 or 72 h (data not shown). Interestingly, no differences in sensitivity to doxorubicin or paclitaxel between the various types of MCF-7 cells were detected (data not shown).

To investigate the influence of signal transduction pathway inhibitors on the sensitivity of MCF-7 cells to cisplatin, inhibitors of PI3-kinase (LY294002), MEK1 (PD98059), or FRAP/mTOR (Rapamycin) were added to MCF-7 cell cultures 1 h prior to 24-h cisplatin treatment, then cell survival was measured. As shown in Fig. 4B, the inhibitors alone moderately decreased cell survival in all MCF-7 clones studied. Likewise, the inhibitors to some extent decreased the survival of cisplatin-treated MCF-7 cells (Fig. 4C). However, MCF-7 CHK(wt) cells were again significantly more resistant to cisplatin treatment. Importantly, none of the inhibitors decreased the survival of cisplatin-treated MCF-7 CHK (wt) cells to the level of the remaining MCF-7 clones, although pre-treatment with LY294002 completely abolished Akt phosphorylation in the cisplatin-treated cells (data not shown). To confirm the results obtained in MCF-7 cells, we screened several breast cancer cell lines (MDA-MB-453, BT-474, T47D, MDA-MD-361, MDA-MB-231, MCF-10A, HBL-100, SK-BR3, UACC-812) for overexpression of Src-family kinases. Although overexpression of Src kinase was quite frequent (data not shown), we did not find any other cell line in which there was a concomitant overexpression of Src and Lyn. We used stable CHK transfectants of the cell line which primarily overexpresses solely Src kinase (MDA-MB-231 cells) and we found no modulation of

resistance to cisplatin in these cells by overexpression of CHK(wt) (data not shown). This result indirectly confirms the role of Lyn in response to cisplatin treatment in breast cancer cells. Interestingly, overexpression of CHK(dk) tended to sensitize MDA-MB-231 cells to cisplatin (data not shown).

## Discussion

Csk homologous kinase (CHK) is a tyrosine kinase, sharing approximately 50% homology with Csk (10). Both kinases were shown to negatively regulate the activity of the Src-family of protein tyrosine kinases by phosphorylating their inhibitory C-terminal tyrosine (e.g. Tyr-529 in Src). Aside from that function, CHK was also shown to associate with the HER-2/neu receptor in heregulin-stimulated breast cancer cells (21,22). As both Src-family kinases and the HER2/neu receptor are closely related to the development of human cancer, the above-mentioned properties of CHK make it a putative candidate as an anti-tumor agent. Indeed, marked inhibition of *in vivo* growth of MCF-7 cells expressing CHK(wt) was observed in our recent studies (16).

Little is known about the role(s) of CHK in normal cellular physiology. It has been shown that the SH2 domain of CHK binds to several tyrosine-phosphorylated proteins that are involved in cell proliferation and differentiation, such as the activated protein tyrosine kinase receptor c-Kit in megakaryocytes (23), the activated protein tyrosine kinase TrkA in PC12 cells (24), as well as the activated protein paxillin in human blastic T cells (25). Although detailed studies on the effect of CHK on major signal transduction

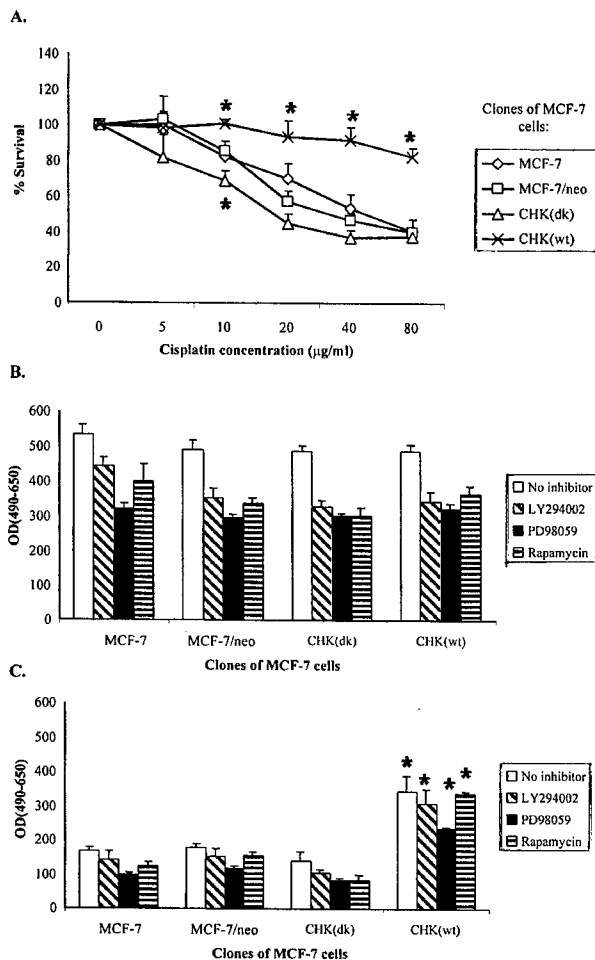


Figure 4. Survival of MCF-7 cells after 24-h treatment with various concentrations of cisplatin (A), and the effects of signal transduction pathway inhibitors on the sensitivity of MCF-7 cells to cisplatin (B and C). (A), MCF-7 cells; parental (MCF-7), transfected with empty vector (MCF-7/neo), and expressing CHK (wt) or (dk), were seeded in triplicate onto 96-well flat-bottomed microtiter plates at a concentration of  $1 \times 10^5$  cells in 200  $\mu$ l. Twenty-four hours after seeding, serial dilutions of cisplatin in 50  $\mu$ l of culture medium were added to the cell cultures. After 24-h incubation, cell survival was assessed by MTT assay as described in Materials and methods. Data represent the means  $\pm$  SE of three independent experiments. (B and C), MCF-7 cells; parental (MCF-7), transfected with empty vector (MCF-7/neo), and expressing CHK (dk) or (wt), were dispensed in a 96-well flat-bottomed microtiter plate at a concentration of  $1 \times 10^5$  cells in 200  $\mu$ l. Twenty-four hours after seeding, LY294002 (final concentration of 25  $\mu$ M), PD98059 (25  $\mu$ M), or Rapamycin (250 nM) were added to the cell cultures. Cells were then incubated for 25 h with the specified inhibitors (B) or 1 h after the addition of the inhibitors, cisplatin was added to the cell cultures (final concentration of 80  $\mu$ g/ml) and the cells were further incubated for 24 h (C). Cell survival was assessed by MTT assay as described in Materials and methods. Data represent the means  $\pm$  SD. \* $P < 0.05$  vs. all other groups in the experiment.

pathways in the cell have not been published yet, it was reported that overexpression of CHK leads to the increased phosphorylation and activation of MAP kinases in PC12 cells stimulated with NGF (24). Thus, we decided to study the effects of overexpressing CHK on the phosphorylation of Akt, the serine/threonine kinase that lies at the crossroads of

multiple cellular signaling pathways. For these studies, we used previously characterized MCF-7 cells stably transfected with the genes encoding CHK(dk) and CHK(wt) (16,21).

Activation of Akt occurs, usually in a PI3-K-dependent manner, by the phosphorylation of Akt on both Thr-308 and Ser-473 (5). As no tyrosine phosphorylation has been shown to directly modulate Akt activity, the putative influence of CHK on Akt would most likely be indirect. It was already shown that Src-family kinases modulate the activity of Akt. However, the manner of this modulation is not the same for all members of the Src-family kinases. It has been shown that active Src kinase induces the activation of Akt (6). In contrast, Lyn kinase was shown in several reports to negatively regulate the activation of Akt. For instance, a several-fold increase in Ser-473 phosphorylation of Akt was reported in *lyn*<sup>-/-</sup> mast cells upon IgE receptor stimulation (7), and BCR crosslinking in Lyn-deficient B cells resulted in markedly enhanced hyperphosphorylation and activation of Akt (8). In our studies using MCF-7 cells, CHK inhibited Lyn activity to a greater extent than it did the activity of Src [Fig. 1B and (16)]. Therefore, we assessed the phosphorylation of Akt in those cells stimulated with two different factors: heregulin or cisplatin. In the experiment with heregulin stimulation, two types of MCF-7 cells were used: parental and those stably transfected with CHK(wt). As shown in Fig. 2, in MCF-7 CHK(wt) cells the heregulin-induced phosphorylation of Akt occurred more rapidly than in the parental cell line, which indicates facilitation of Akt-mediated signal transduction by the overexpression of CHK(wt). To confirm this assumption, we used another agent inducing activation of Akt-cisplatin. For this experiment, four different types of MCF-7 cells were studied: parental (MCF-7), transfected with empty vector (neo), and expressing CHK dead-kinase (dk) or wild-type (wt). Again, more rapid and also stronger phosphorylation was observed in MCF-7 cells overexpressing CHK(wt) in comparison to other cell types used in the experiment. Additionally, the phosphorylation of Akt in cells overexpressing CHK(dk) tended to be weaker than in the parental or neo cells (Fig. 3B).

As it was suggested that the Akt-mediated survival pathway may be an important contributor to cancer chemoresistance (26,27), our next step was to investigate the sensitivity of MCF-7 cells to cisplatin treatment. As shown in Fig. 4, MCF-7 cells overexpressing CHK(wt) were markedly more resistant to cisplatin than the three remaining cell types. Moreover, MCF-7 cells expressing CHK(dk) were more sensitive to cisplatin than were the parental or neo MCF-7 cells. These results correlate with the extent of Akt Ser-473 phosphorylation after cisplatin treatment (Fig. 3), suggesting that the increase in Akt activity might protect the cells from the cytotoxic influence of cisplatin. Also, these observations are in accordance with the most recent results obtained by Asselin *et al* in an ovarian cancer model, as the authors of that study proposed the involvement of the Akt survival pathway in the XIAP-mediated resistance to cisplatin (20). Despite that similarity, a substantial difference exists between the influences of overexpressing XIAP versus CHK on Akt phosphorylation. XIAP caused an increase in Akt phosphorylation in quiescent cells, while overexpression of CHK in our model seemed to facilitate this process in



response to other stimuli. Thus, although the co-existence of increased Akt phosphorylation and resistance to cisplatin is clear in both models, the exact mechanism of that resistance is not known and remains to be elucidated in further studies. Interestingly, inhibitors of either PI3-K-mediated (LY294002, Rapamycin) or MAPK-mediated (PD98059) pathways failed to abolish the resistance to cisplatin of the MCF-7 cells expressing CHK(wt). This observation suggests the influence of CHK overexpression on other processes than those identified to date, namely the activation of MAP kinases (24) or the PI3-K-mediated phosphorylation of Akt as described here.

In conclusion, our results suggest that in MCF-7 cells overexpressing CHK kinase, Akt becomes more easily phosphorylated in response to growth factor (heregulin) or DNA damaging agent (cisplatin) treatment. Although CHK-mediated facilitation of Akt phosphorylation correlated with an imbalance in inhibition of Src and Lyn kinases, the cellular mechanism(s) of this phenomenon are unknown.

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# Carboxyl-Terminal Src Kinase Homologous Kinase Negatively Regulates the Chemokine Receptor CXCR4 through YY1 and Impairs CXCR4/CXCL12 (SDF-1 $\alpha$ )-Mediated Breast Cancer Cell Migration

Byeong-Chel Lee,<sup>1</sup> Tae-Hee Lee,<sup>1</sup> Radoslaw Zagodzdzon,<sup>1</sup> Shalom Avraham,<sup>1</sup> Anny Usheva,<sup>2</sup> and Hava Karsenty Avraham<sup>1</sup>

Divisions of <sup>1</sup>Experimental Medicine and <sup>2</sup>Endocrinology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts

## Abstract

Using microarray gene analysis, we found that carboxyl-terminal Src kinase homologous kinase (CHK) regulated the expression of the chemokine receptor, CXCR4. Northern blot and fluorescence-activated cell-sorting analyses showed that CHK down-regulated CXCR4 mRNA and protein levels, respectively. Mutated CHK, which contains a mutation within the ATP binding site of CHK, failed to inhibit CXCR4 expression, thus suggesting that CHK kinase activity is involved in the regulation of CXCR4. Results from gel shift analysis indicated that CHK regulates CXCR4 transcriptional activity by altering YY1 binding to the CXCR4 promoter. Whereas CHK had no significant effects on the expression of YY1, c-Myc, Max, and other YY1-binding proteins, CHK was found to modulate the YY1/c-Myc association. Furthermore, CHK inhibited CXCR4-positive breast cancer cell migration. Taken together, these studies show a novel mechanism by which CHK down-regulates CXCR4 through the YY1 transcription factor, leading to decreased CXCR4-mediated breast cancer cell motility and migration. (Cancer Res 2005; 65(7): 2840-5)

## Introduction

The carboxyl-terminal Src kinase (Csk) family of non-receptor-type tyrosine kinases consists of Csk and the Csk homologous kinase, CHK (1). Despite their structural similarities, Csk homologous kinase (CHK) and Csk show striking differences. Whereas Csk is ubiquitously expressed, CHK expression is limited to brain and hematopoietic cells. Moreover, whereas CHK knockout mice do not show any apparent abnormal phenotype, Csk knockout mice present with a defect in neural tube formation and death at E11.5 (2). CHK has been suggested to play a specific role as a novel negative growth regulator of human breast cancer based on the following observations: (a) Unlike Csk, which is ubiquitously expressed, CHK is specifically expressed in primary breast cancer specimens but not in normal breast tissues (3-5). (b) Unlike Csk, which cannot associate with ErbB-2, CHK binds directly to phospho-Tyr<sup>1248</sup> of the ErbB-2/neu receptor kinase upon heregulin stimulation and inhibits Src kinase activity (4). (c) Overexpression of CHK in breast cancer cells markedly inhibits the cell growth, transformation, and invasion induced by heregulin and causes a

significant delay of cell entry into mitosis (5). Thus, CHK not only inhibits breast cancer cell proliferation and transformation but also may inhibit tumor cell invasion, suggesting its possible role in cell motility and metastasis in breast cancer.

There is now accumulating evidence of a role for chemokines and their receptors in cancer cell motility and metastasis (6-10). Whereas most chemokine/chemokine receptor studies have focused on leukocytes, recent studies have highlighted another important role for chemokines/chemokine receptors in the metastasis of breast cancer, especially showing CXCR4 as an important mediator of breast cancer metastasis (9). These results indicate that the elevated expression of CXCR4 is correlated with a more metastatic breast cancer cell phenotype. The potential contribution of CXCR4 to cancer metastasis is further supported by recent studies demonstrating a high level of CXCR4 expression in malignant ovarian cancers and glioblastomas (11, 12).

YY1 is a 65-kDa DNA-binding protein, which can either repress or activate transcription depending on the context (13). YY1 represses the promoter activity of CXCR4 through its binding to the upstream region of the CXCR4 promoter (14, 15).

In this study, we identified CHK as a negative regulator of CXCR4, which is an important signature molecule in breast cancer metastasis. CHK down-regulated the level of CXCR4 expression through YY1, a potent negative regulator of CXCR4. This suppressive effect was attenuated by a mutation at position 262 in the ATP-binding site of the kinase domain of CHK (kinase-dead CHK), suggesting that CHK kinase activity is involved in this regulatory mechanism. We also showed that CHK expression impaired the transmigration of breast cancer cells *in vitro*. This study provides novel insights into the regulation of CXCR4 by the nonreceptor tyrosine kinase, CHK.

## Materials and Methods

**Materials.** Antibodies and reagents were obtained from the following sources: polyclonal anti-YY1 (H414), polyclonal anti-CSK (c-20), polyclonal anti-Lsk, polyclonal p300, and monoclonal CBP were from Santa Cruz Biotechnology (Santa Cruz, CA). pGL-CXCR4 (-375) was a generous gift from Dr. Moriuchi (Division of Medical Virology, Nagasaki University Hospital, Nagasaki, Japan) and contains CXCR4 between -357 and +51 relative to the transcription site followed by the luciferase gene (14).

**Cell culture and transfections.** MDA-MB-231 cells were cultured in DMEM with 10% FCS. MCF-7 cells were grown in RPMI containing 10% FCS. These cell lines were cultured at 37°C in 5% CO<sub>2</sub>. Transfections were done using LipofectAMINE Plus according to the manufacturer's instructions (Life Technologies, Inc, Grand Island, NY). For stable clones, the transfectants were selected with G418 (Life Technologies). The expression of CHK in the G418 selected clones was confirmed by immunoblotting with anti-Lsk polyclonal antibody (Santa Cruz Biotechnology).

**Fluorescence-activated cell-sorting analysis.** Cells were briefly trypsinized and resuspended in PBS supplemented with 0.1% bovine serum

**Note:** B. Lee and T. Lee contributed equally to this work.

**Requests for reprints:** Hava Karsenty Avraham, Division of Experimental Medicine, Beth Israel Deaconess Medical Center, Harvard Institutes of Medicine, 4 Blackfan Circle, 3rd Floor, Boston, MA 02115. Phone: 617-667-0073; Fax: 617-975-6373; E-mail: havraham@bidmc.harvard.edu.

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albumin. Cells were incubated with anti-CXCR4 monoclonal antibody (clone 12G5, R&D Systems, Minneapolis, MN) for 30 minutes on ice followed by a 30 minutes incubation on ice with anti-mouse secondary antibody conjugated to FITC or TRITC (Jackson ImmunoResearch, West Grove, PA). After the final wash, cells were fixed in 1% paraformaldehyde and analyzed using a FACScan (Becton Dickinson, San Jose, CA).

**Chemotaxis assay.** The chemotaxis assay was done as described (16). Briefly, MDA-MB-231 cells were starved overnight in serum-free medium before application to an 8- $\mu$ m pore-size fibronectin (50  $\mu$ g/mL)-coated transwell insert (Costar, Cambridge, MA). Cells were suspended into the upper chamber at a final concentration of  $50 \times 10^4$  cells/mL in 200  $\mu$ L of RPMI 1640, and then recombinant CXCL12 (SDF-1 $\alpha$ , 10–20 ng/mL) was added to the lower chamber. After 3 to 6 hours of incubation, the cells on the upper surface of the filters were removed by wiping with cotton swabs, and the migrated cells on the lower chamber were fixed and stained using a Hema3 kit (Biochemical Sciences Inc, Swedesboro, NJ) according to the manufacturer's instructions. Cellular transmigration was enumerated in four separate microscopic fields per field.

**Western blot analysis and immunoprecipitation.** Western blot analysis and immunoprecipitation were done as described (17).

**Electrophoretic mobility shift assay.** Nuclear extracts were prepared using MCF-7 cells as described (15). Complementary oligonucleotides corresponding to YY1 (5'-TAGCAAGGATGGACGCGCCACAGAGAGAC-3') were annealed and end-labeled with [ $\gamma$ - $^{32}$ P]ATP. Competition experiments were done with 1:50 ratios of labeled to nonlabeled oligonucleotides. The binding reactions were done and analyzed on 5% nondenaturing gel as described (15). For the interference assay, 1  $\mu$ L of YY1 polyclonal antibody (H414, Santa Cruz Biotechnology) was preincubated with the cell extracts before addition of the appropriate radiolabeled oligonucleotide.

**Luciferase assay.** Cells were plated at  $(2 \text{ to } 3) \times 10^5$  cells per 60-mm dish 1 day before transfection with the promoter-luciferase construct. Cells were transfected with LipofectAMINE (Life Technologies, Rockville, MD) and with the reporter constructs, CHK and  $\beta$ -galactosidase. After a 24-hour transfection, cells were harvested for the luciferase assay. Luciferase activity was measured using the Luciferase assay system (Promega, Madison, WI). In all of the cotransfection experiments, transfection efficiency was normalized by assaying  $\beta$ -galactosidase activity using the  $\beta$ -galactosidase gene under the control of the SV40 early promoter as an internal control.

**Northern blot analysis and quantitation of CXCR4 mRNA.** Total RNAs were prepared from MDA-MB-231 and MCF-7 cells using RNASat60 reagent (Tel-Test, Friendswood, TX). Twenty micrograms of each RNA sample were electrophoresed on a 1% formamide-agarose gel and transferred to nylon membranes (Hybond-N+, Amersham Biosciences, Piscataway, NJ). The membranes were cross-linked under UV light, prehybridized, and then hybridized in Expresshyb hybridization solution (Clontech, Palo Alto, CA). The hybridized membranes were exposed to X-ray film at  $-80^\circ\text{C}$ . CXCR4 mRNA of individual stable clones was quantified using the biotin-labeled CXCR4 oligonucleotide probe and standard provided in the Quantikine mRNA probes and calibrator kit (R&D Systems) according to the manufacturer's instructions. The minimum detectable dose of the kit ranged from 0.78 to 4.0 amol/mL.

**Microarray analysis.** The Human Cytokine Expression array was purchased from R&D Systems. The membrane consists of 847 genes, which includes cytokines, chemokines, and other immunomodulatory factors, as well as the receptors for these categories of molecules. Probe synthesis and hybridization were done according to the manufacturer's specifications. Quantitation of array images was determined using the ArrayVision software (Imaging Research, Inc., Ontario, Canada).

## Results

**Regulation of CXCR4 expression by CHK.** Our previous results indicated that a nonreceptor tyrosine kinase, CHK, inhibits the invasion/migration of breast tumor cells *in vitro* (5). Thus, we sought to determine whether CHK inhibits breast cancer cell

invasion/migration by modulating chemokines, chemokine receptors, or cytokines because chemokines and their receptors have emerged as key molecules implicated in breast cancer cell migration and metastasis (6, 9, 18). Using cDNA probes derived from MDA-MB-231 cells transfected with CHK or with control vector, we did microarray analysis. The array represents a comprehensive collection of cytokines, chemokines, and other immunomodulatory factors and their receptors. Whereas most metastasis-related genes, including matrix metalloproteinases (MMP) and integrins, did not show significant changes in their expression, CXCR4 was found to be significantly suppressed by CHK (Fig. 1A). To confirm the microarray results, mRNA extracted from CHK stable transfectants of the MDA-MB-231 and MCF-7 clones was analyzed by Northern blot analysis. The expression of CXCR4 mRNA was suppressed in wild-type CHK-transfected cell lines (W1 and W2 of MCF-7 stable clones and W1 of the MDA-MB-231 stable clone; Fig. 1B). Interestingly, enhanced CXCR4 mRNA expression was observed in mutant CHK (A262G, ATP binding site) transfected clones (M1 and M2 of MCF-7 clones and M1 of MDA-MB-231 clones), suggesting that CHK kinase activity might be involved in the transcriptional regulation of CXCR4. The CXCR4 regulation shown is not due to the homologous kinase, Csk, because our Western blot (Fig. 1C) and Northern blot (data not shown) analyses did not show any differences in Csk expression between the mock- and CHK-transfected clones. To confirm that these regulatory effects of CHK were not due to clonal variation, we established additional stable clones in MDA-MB-231 cells ( $n = 7$ ), and quantitated the CXCR4 mRNA levels. Most of the clones tested showed an  $\sim 50\%$  to  $60\%$  decrease in CXCR4 mRNA (Fig. 1D). To analyze whether CHK regulates CXCR4 transcriptional activity, we transfected CXCR4-luciferase reporter constructs into MDA-MB-231 cells, and then assessed the effects of CHK. The presence of the wild-type CHK caused a significant reduction (at least 3-fold) in CXCR4 promoter activity in both transiently transfected and stably cloned MDA-MB-231 cells as compared with the mock-transfected cells (vector; Fig. 1E). We also found similar effects of wild-type CHK in the MCF-7 cells (data not shown), although the extent of suppression was not as significant as in the MDA-MB-231 cells.

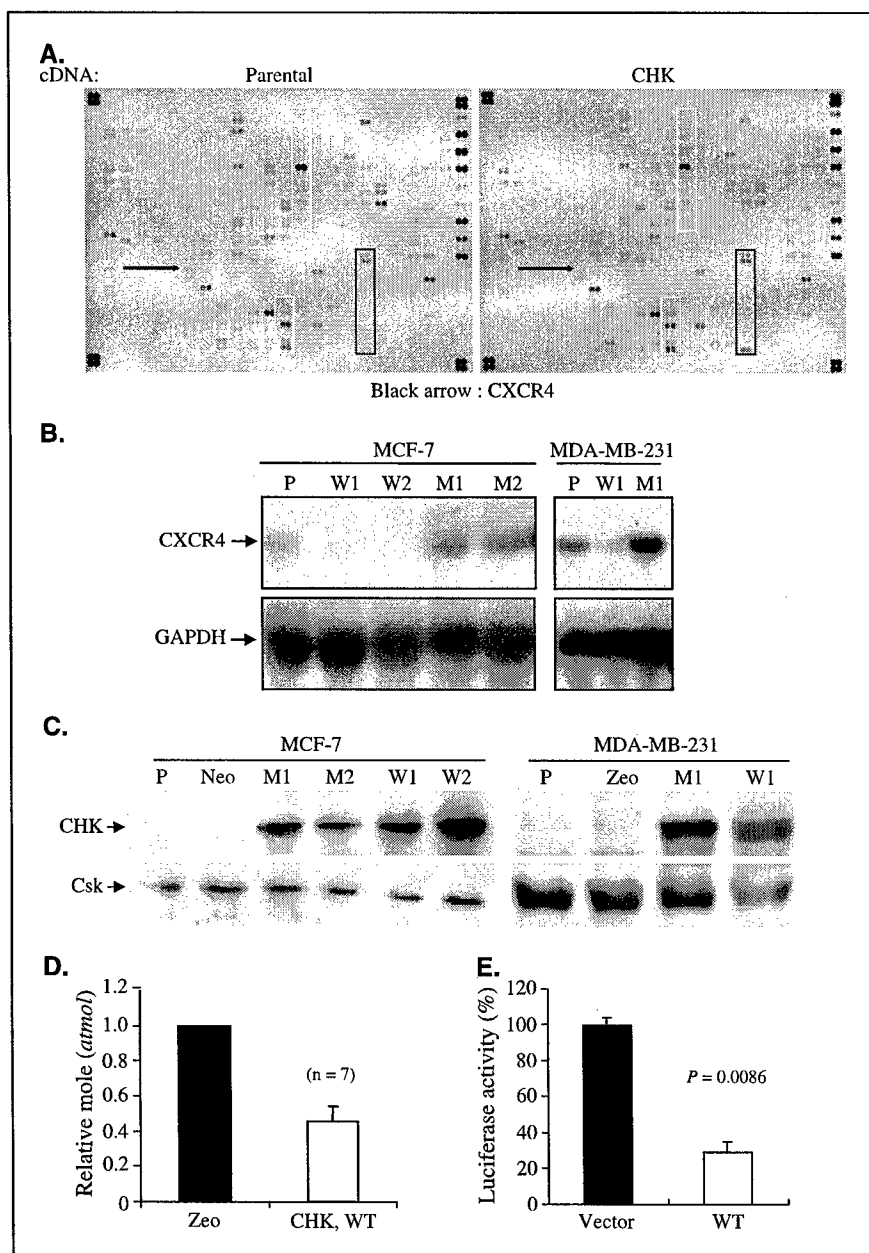
**CHK mediated CXCR4 regulation via YY1.** To understand more fully the molecular mechanism of CHK-induced transcriptional repression of the CXCR4 promoter, we embarked on studies of the cellular DNA-binding proteins that interact with the CXCR4 promoter elements. The CXCR4 promoter contains several cis-acting regulatory elements including E box family proteins and a YY1 binding site (14). YY1 represses the promoter activity of CXCR4 through its binding to the upstream region of the CXCR4 promoter (14, 15). Therefore, we examined the possibility of whether a similar regulatory mechanism is also involved in CHK-mediated CXCR4 modulation. Stably transfected MCF-7 cells were harvested, and the binding of YY1 to the CXCR4 promoter was visualized by electrophoretic mobility shift assay. As shown in Fig. 2, when analyzed with nuclear extracts from stably transfected MCF-7 cells, a sequence-specific DNA protein complex was detected. The formation of the complex corresponding to YY1 binding was competed by an excess of the YY1-unlabeled probe, but not by an unrelated double-stranded oligonucleotide, AP1. In the presence of anti-YY1 antibody, but not control normal rabbit immunoglobulin (NRIgG), the YY1 complex was disrupted, demonstrating that the indicated band was due to the binding of YY1. This suggests that the observed

binding activity resulted from the specific binding of YY1 in the extracts to the DNA probe. Interestingly, mutation in the ATP binding site sequence of CHK alleviated YY1 binding to the CXCR4 promoter elements (Fig. 2, lane 3).

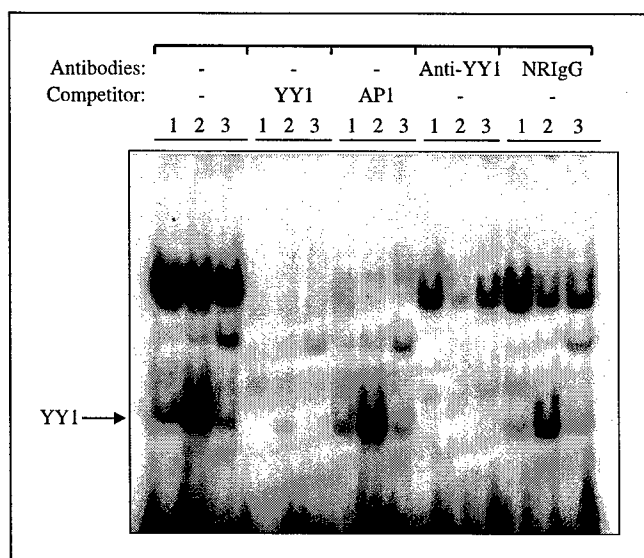
**CHK has no effects on YY1, c-Myc, or Max expression but down-regulates CXCR4 expression via modulation of the YY1-c-Myc interaction.** c-Myc associates with YY1, which mutually inhibits their biological functions (19). Overexpression of c-Myc protein is often found in poorly differentiated and highly proliferative breast cancer cells (18–21). We therefore analyzed whether CHK down-regulates c-Myc in breast cancer, reducing YY1/c-Myc complexes, and thus allowing more YY1 to bind to the CXCR4 promoter. To test this hypothesis, we measured the level of c-Myc in the stable clones. As shown in Fig. 3A (top), CHK had no significant effect on the expression of c-Myc. In addition, the ectopic expression of CHK did not have any direct effects on the

endogenous level of YY1 as shown in Fig. 3A (middle). Although Max does not directly associate with YY1, it does associate with c-Myc (22). Thus, changes in Max levels could alter the Max/c-Myc association followed by alteration of the c-Myc and YY1 association. However, as shown in Fig. 3A (bottom), CHK had no significant effect on the expression of Max.

Previous studies showed that human herpesvirus 6 (HHV-6) infected cells alter the association of c-Myc with YY1, thus up- or down-regulating CXCR4 promoter activity (15). We therefore assessed whether CHK regulates the interaction between YY1 and c-Myc. To test whether CHK regulates the YY1/c-Myc association, 293T cells were cotransfected with wild-type or mutant CHK together with c-Myc, and then analyzed for c-Myc and YY1 association. As shown in Fig. 3B, the levels of c-Myc associated with YY1 were lower in the wild-type CHK-transfected cells as compared with the mutant CHK-transfected cells. The endogenous

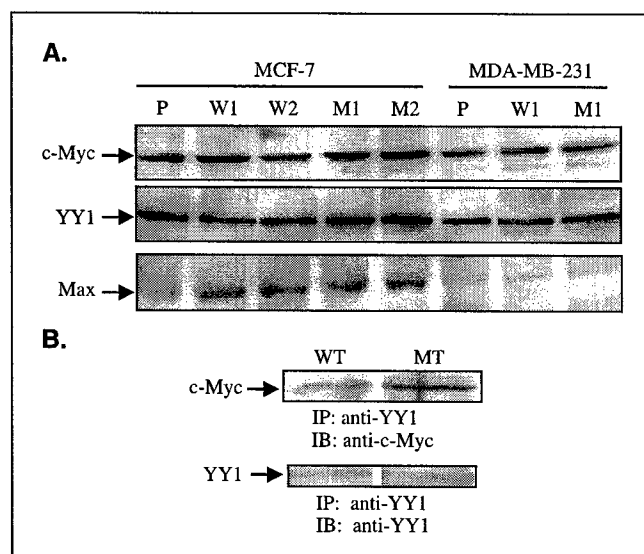


**Figure 1.** A, cDNA microarray analysis of parental and CHK-transfected MDA-MB-231 breast cancer cells. Gene expression was measured by cDNA array hybridization (Human Cytokine Array, R&D Systems) of RNA from each of the indicated cell lines. Arrow, indicates the location of CXCR4. The locations of integrin ( $\square$ ) and MMPs ( $\blacksquare$ ) are highlighted. B, Northern blot analysis. Total RNA (20  $\mu$ g) was size-fractionated, blotted onto a nylon membrane, and then hybridized to the  $^{32}$ P-labeled CXCR4 cDNA probe. Hybridization with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a control for RNA loading (bottom). P, parental cell line; W, wild-type CHK; M, mutant CHK. Number, independent clone. C, CHK expression in stably transfected MDA-MB-231 and MCF-7 breast cancer cells. Whole-cell lysates were probed for CHK by Western blotting. Lanes, P, parental cell line; Neo and Zeo, mock-transfected; M, kinase-dead CHK-transfected; W, wild-type CHK-transfected. Number, independent clone. Note that Csk expression was not affected by ectopic CHK expression. D, quantitation of human CXCR4 mRNA in several independent MDA-MB-231 stable clones. Stable clones were selected in the presence of G418. Total RNA was extracted from each clone ( $n = 7$ ) and analyzed for the quantitation of CXCR4 mRNA using a Quantikine CXCR4 mRNA kit (R&D Systems). Zeo, mock-transfected cells. E, CHK (WT) represses CXCR4 promoter activity. The effects of wild-type CHK (WT) on CXCR4 promoter activity were measured in MDA-MB-231 breast cancer cells. Cells were cotransfected with CXCR4 promoter luciferase constructs and the wild-type CHK expression vector (WT). Twenty-four hours after transfection, luciferase assays were done. Results are expressed as the percent of luciferase activity, which was calculated relative to the activity of the same reporter construct cotransfected with an empty expression vector. Representative of four independent experiments with similar results. Columns, mean of three experiments; bars, SD. P value is shown in the upper right corner.

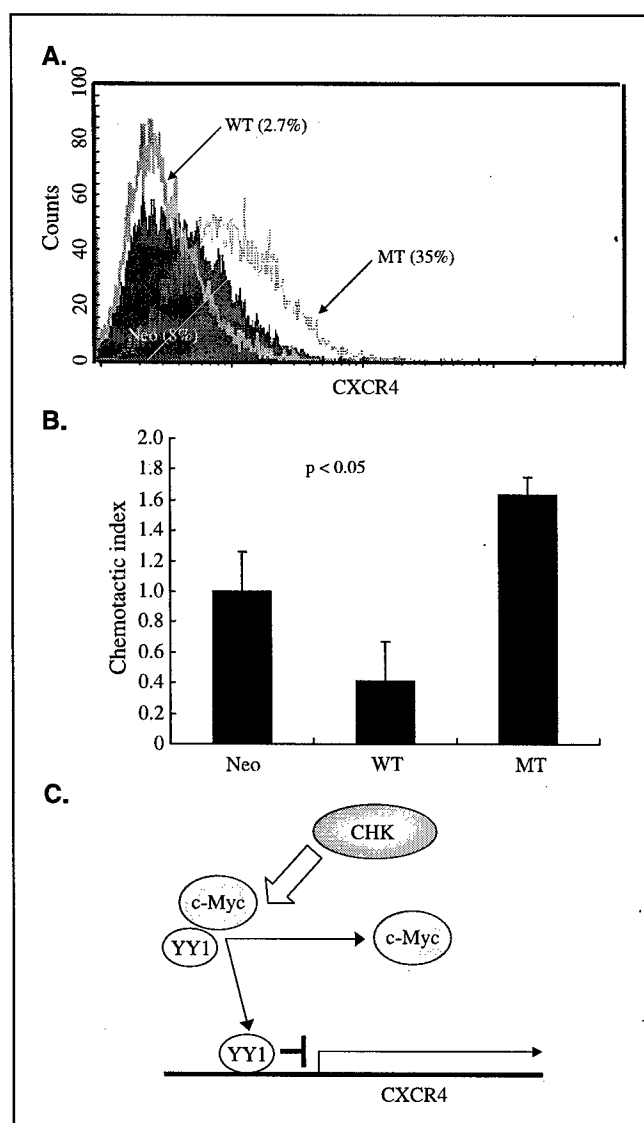


**Figure 2.** CHK regulates YY1 binding to CXCR4 promoter elements. **A**,  $^{32}$ P-end-labeled oligonucleotide, corresponding to the sequence from -49 to -71 of the CXCR4 promoter, was incubated with the nuclear cell extracts of MCF-7 stable clones: 1, vector alone; 2, wild-type CHK; 3, mutant CHK. The specificity of the complexes was assessed by competition experiments using a 50-fold molar excess of the corresponding unlabeled YY1 oligonucleotide or an unrelated oligonucleotide (AP1). Arrow, YY1 complexes. The YY1 complex was disrupted by the anti-YY1 polyclonal antibody (anti-YY1), but not by the normal rabbit immunoglobulin (NRIgG). Representative results from three independent experiments.

association of YY1/c-Myc was also measured using stable clones, yielding similar results (data not shown). Thus, CHK regulates CXCR4 transcriptional activity by altering the physical association of YY1/c-Myc.



**Figure 3.** CHK does not affect the protein synthesis of YY1, c-Myc, or Max, but alters the *in vivo* association of c-Myc with YY1. **A**, the amount of YY1, c-Myc, or Max in the stable clones was determined by immunoblotting with their corresponding antibodies. Representative of five independent experiments with similar results. **B**, YY1 was immunoprecipitated from the whole-cell lysates of wild-type (WT) or mutant (MT) CHK-transfected MDA-MB-231 cells using anti-YY1 antibody. The immunoprecipitates (IP) were then analyzed by immunoblot analysis (IB) with anti-c-Myc antibodies (top). For the loading control, the level of YY1 protein in the immunoprecipitated cell lysates was determined with anti-YY1 antibody (bottom). Representative of three independent experiments with similar results.



**Figure 4.** CHK overexpression impaired transmigration of the CXCR4-positive breast cancer cell line, MDA-MB-231, toward CXCL12. **A**, cell surface expression of CXCR4 in stably transfected MDA-MB-231 cells. CXCR4 expression was determined as described in Materials and Methods. Representative of three independent experiments with similar results. For the histograms: Neo, mock-infected; WT, wild-type CHK-infected; MT, mutant CHK-transfected. **B**, cell migration was evaluated using fibronectin-coated 8- $\mu$ m transwells. Migration of the cells was enumerated as described in Materials and Methods. Representative of three independent experiments with similar results. Results are shown as the mean  $\pm$  SD. The statistical significance was analyzed using the Student's *t* test (two-tailed distribution). Differences were considered to be significant when  $P < 0.05$ . **C**, model for CXCR4 repression mediated by CHK. CHK alters the association of c-Myc with YY1, thus interfering with the ability of YY1 to regulate CXCR4 transcription.

**Effects of CHK on the migration of breast cancer cells.** Next, we tested whether CXCR4 expression is also regulated at the protein level. As the functionality of CXCR4 is correlated with its expression on the cell surface, stable clones were analyzed for their cell surface CXCR4 expression. Similar to the Northern blot analysis results, CXCR4 protein expression was decreased in wild-type CHK-transfected MDA-MB-231 cells (Fig. 4A). Whereas MDA-MB-231 cells transfected with wild-type CHK showed a significant decrease from 8% to 2.7% in CXCR4 protein expression, as compared with

the mock-transfected cells (neo), MDA-MB-231 cells transfected with mutant CHK expressed more CXCR4, with expression increasing from 8% to 35%. These results show that CHK not only regulates CXCR4 at the transcriptional level but also regulates CXCR4 at the protein level. To define the role of CHK in breast cancer cell migration, we carried out a transmigration assay of stably transfected breast cancer cell lines in the presence of CXCL12 (SDF-1). Because MDA-MB-231 cells have been widely used to study breast cancer cell migration and *in vivo* metastasis, we used MDA-MB-231 cells for the transmigration assay. Whereas wild-type CHK inhibited MDA-MB-231 cell migration toward CXCL12 as compared with the mock-transfected cells (~60% inhibition), mutant CHK increased the migration of the cells (Fig. 4B). We did not observe any differential chemokinesis between the clones that we tested (data not shown).

## Discussion

CXCR4 expression was found to be correlated with the malignant progression of tumors (10, 12, 23, 24). A recent study showed that expression of the chemokine receptor, CXCR4, is tightly correlated with the metastatic properties of breast cancer cells (8, 9).

Whereas CHK exerts its tumor suppressive effects by down-regulating ErbB-2/neu-activated Src kinases (5), our present study revealed another novel link between CHK and CXCR4. Northern blot analysis, a promoter assay, as well as fluorescence-activated cell-sorting (FACS) analysis, showed that wild-type CHK down-regulated the level of CXCR4 in human breast cancer cells. Interestingly, point mutation at the ATP-binding site of the CHK kinase domain attenuated the suppressive effect of CHK on CXCR4 expression, suggesting that its kinase activity is involved in this regulatory mechanism.

Previous studies showed that a nonreceptor tyrosine kinase, ZAP-70, regulates the transmigration of T lymphocytes toward CXCL12 (25, 26). ZAP-70 is a protein tyrosine kinase that associates with the  $\zeta$  subunit of the T-cell antigen receptor and undergoes tyrosine phosphorylation following T-cell receptor stimulation (27, 28). The Tyr<sup>292</sup> in the interdomain B region of ZAP-70 negatively regulates ZAP-70-dependent effects on T-cell migration. More recently, the upstream molecule, Lck, was also shown to regulate the T-cell chemotaxis induced by CXCL12, suggesting that the ZAP-70/Lck signaling pathway may have a critical role in T-lymphocyte migration (29). However, the lower migration of ZAP-70- and Lck-deficient cells was not due to the regulation of CXCR4 receptor expression, as determined by FACS analysis.

Although cytokines, including interleukin 2 (30), transforming growth factor  $\beta$  and IFN- $\gamma$  (31, 32), have been shown to modulate the level of CXCR4 expression, the detailed mechanisms involved

in the regulation of its expression are unknown. Moreover, all of these studies were done in T lymphocytes.

Increased Src activity is frequently associated with the metastasis of cancer cells. Src activation by SDF-1 $\alpha$  also contributes to this metastasis (33–35). Src is one of the downstream molecules of CHK, and its kinase activity is often increased significantly in advanced cancer (36). Here, we have tested whether Src is involved in the regulation of CXCR4 expression. However, the repression of CXCR4 is not likely due to negative regulation of Src kinase activity because the treatment of breast cancer cell lines with Src kinase inhibitors did not show significant effects on CXCR4 transcription.<sup>3</sup>

Rather than the factors described above, our study revealed a novel pathway by which CHK negatively down-regulates CXCR4, through the transcriptional factor YY1. YY1 binds to the upstream region of the CXCR4 promoter and negatively regulates CXCR4 promoter activity (14). Our gel shift assay indicated that wild-type CHK regulates the DNA binding activity of YY1. Furthermore, mutated CHK attenuated the ability of YY1 to bind DNA, suggesting that the kinase activity of CHK plays a role in modulating the DNA-binding ability of YY1. Although CHK does not modulate the level of YY1, c-Myc, or Max, our results suggest that CHK affects c-Myc and YY1 association by a mechanism similar to that shown in HHV-6-infected T lymphocytes (15). Our findings are summarized in Fig. 4C. In addition, we have tested whether CHK regulates other YY1-binding proteins, such as CBP and p300. We found that CHK does not show any effects either on the levels of CBP and p300 or on their association with YY1.<sup>3</sup> The ectopic expression of CHK in the metastatic breast cancer cell line, MDA-MB-231, significantly impaired its ability in the *in vitro* transmigration assay, suggesting the potential role of CHK in regulating tumor cell motility and metastasis.

Taken together, our study shows a novel mechanism by which CHK regulates the level of CXCR4, through the YY1 transcription factor, and impairs CXCR4/CXCL12-mediated breast cancer cell migration.

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This article is dedicated to Charlene Engelhard for her continuing friendship and support of our research program.

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<sup>3</sup> Lee et al, unpublished data.

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## Csk Homologous Kinase (CHK) and ErbB-2 Interactions Are Directly Coupled with CHK Negative Growth Regulatory Function in Breast Cancer\*

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Soyoun Kim‡, Radoslaw Zagodzón‡§, Alan Meisler¶, James D. Baleja¶, Yigong Fu‡, Shalom Avraham‡, and Hava Avraham‡¶

From the ‡Division of Experimental Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02115 and the ¶Department of Biochemistry, Tufts University School of Medicine, Boston, Massachusetts 02111

Our previous studies demonstrated that Csk homologous kinase (CHK) acts as a negative growth regulator of human breast cancer through inhibition of ErbB-2/neu-mediated Src family kinase activity (Bougeret, C., Jiang, S., Keydar, I., and Avraham, H. (2001) *J. Biol. Chem.* 276, 33711–33720. The interaction between the CHK SH2 domain and Tyr(P)<sup>1248</sup> of the ErbB-2 receptor has been shown to be specific and critical for CHK function. In this report, we investigated whether the interaction of the CHK SH2 domain and ErbB-2 is directly related to the inhibition of heregulin-stimulated Src kinase activity. We constructed three CHK SH2 domain binding mutants: G129R (enhanced binding), R147K (inhibited binding), and R147A (disrupted binding). NMR spectra for the domains of each construct were used to evaluate their interaction with a Tyr(P)<sup>1248</sup>-containing ErbB-2 peptide. G129R showed enhanced binding to ErbB-2, whereas binding was completely disrupted by R147A. The enhanced binding mutant showed chemical shift changes at the same residues as wild-type CHK, indicating that this mutant has the same binding characteristics as the wild-type protein. Furthermore, inhibition of heregulin-stimulated Src kinase activity was markedly diminished by R147A, whereas G129R-mediated inhibition was stronger as compared with wild-type CHK. These results indicate that the specific interaction of CHK and ErbB-2 via the SH2 domain of CHK is directly related to the growth inhibitory effects of CHK. These new CHK high affinity binding constructs may serve as good candidates for inhibition of the ErbB-2/Src transduction pathway in gene therapy studies in breast cancer.

The majority of breast carcinomas appear to be sporadic and have a complex accumulation of molecular and cellular abnor-

malities that constitute the malignant phenotype (1, 2). In many cases, the random onset of breast cancer correlates with overexpression of the ErbB-2/neu receptor and Src tyrosine kinase activity (3, 4). Downstream activation by the ErbB-2/neu receptor involves intracellular pathways mediated by Ras/mitogen-activated protein kinase, phosphatidylinositol 3-kinase, and phospholipase C $\gamma$ ; however, the molecular mechanisms of these processes are poorly understood (5). Src tyrosine kinase has been suggested to be a main downstream activator of the ErbB-2/neu receptor because the increased Src kinase activity observed in ErbB-2/neu-induced tumors results from the ability of the Src SH2<sup>1</sup> domain to interact directly with ErbB-2/neu in a phosphotyrosine-independent manner (6, 7). Once the ErbB-2/neu receptor is activated by heregulin, it undergoes autophosphorylation at five tyrosine residues located in its non-catalytic carboxyl terminus. The autophosphorylation of ErbB-2/neu can also be induced in the absence of any ligand by high level overexpression of ErbB-2/neu protein (8), as occurs in BT474 or MDA-MB-361 cells.<sup>2</sup> The autophosphorylated tyrosine residues provide docking sites for proteins to connect to intracellular pathways (9, 10). The individual target and effect of each phosphotyrosine are not clear, but an add-back mutation study showed that autophosphorylation of tyrosine residues is involved in both the positive and negative effects on ErbB-2/neu-mediated transformation (11). Tyr<sup>1248</sup> of ErbB-2/neu, which is conserved between human and rodent ErbB-2/neu, has been suggested to be the most critical residue for the oncogenicity of the constitutively activated receptor (12). Thus, the study of proteins that bind to Tyr(P)<sup>1248</sup> of ErbB-2/neu is important in elucidating ErbB-2/neu-mediated signaling and function in cancer development.

The Csk homologous kinase (CHK) protein comprises SH3, SH2, and tyrosine kinase domains. Its SH2 domain interacts with Tyr(P)<sup>1248</sup> of the ErbB-2/neu receptor in a ligand- and receptor-specific manner (13). CHK, like Csk, down-regulates Src kinase activity by phosphorylation of the conserved tyrosine residue in the carboxyl terminus of Src-related enzymes *in vitro*. However, CHK has been suggested to play a specific role as a novel negative growth regulator of human breast cancer on the basis of the following observations. 1) Unlike Csk, which is ubiquitously expressed and cannot associate with ErbB-2, CHK is specifically expressed in primary breast cancer specimens, but not in normal breast tissues (13–15). CHK expression in normal tissues is restricted to hematopoietic cells and brain (16–18). 2) CHK binds directly to Tyr(P)<sup>1248</sup> of the ErbB-2/neu receptor kinase upon heregulin stimulation and inhibits Src

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¶ To whom correspondence should be addressed: Div. of Experimental Medicine, Beth Israel Deaconess Medical Center, Harvard Institutes of Medicine, 4 Blackfan Circle, Boston, MA 02115. Tel.: 617-667-0073; Fax: 617-975-6373; E-mail: havraham@caregroup.harvard.edu.

<sup>1</sup> The abbreviations used are: SH, Src homology; CHK, Csk homologous kinase; GST, glutathione S-transferase; EGFP, enhanced green fluorescent protein; HSQC, heteronuclear single quantum correlation.

<sup>2</sup> R. Zagodzón and H. Avraham, unpublished data.



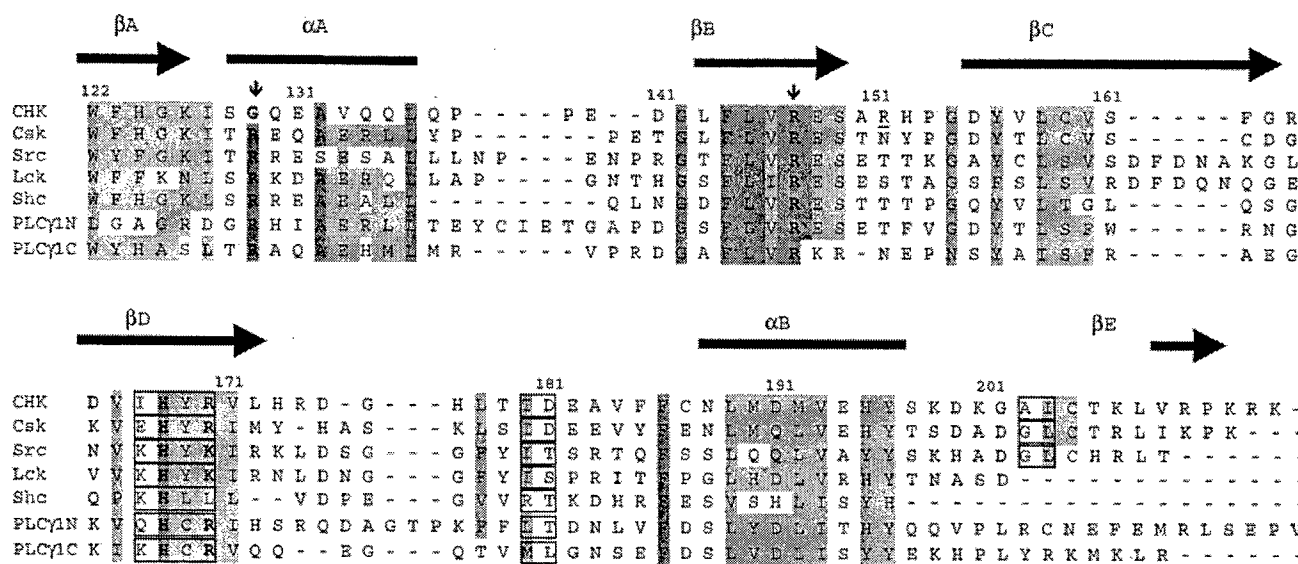


FIG. 1. **Structural alignment of several SH2 domains.** The SH2 domains of CHK, Csk, Src, Lck, Shc, and phospholipase Cγ1 (PLCγ1) were aligned using the T-COFFEE program (available at [ch.embnet.org](http://ch.embnet.org)). Solid bars above the amino acid sequences indicate the secondary structural elements. Strictly conserved residues are shown in dark gray, and moderately conserved residues are shown in light gray. Residues involved in the interaction with Tyr(P) are shown in boldface, and residues that contribute to the interactions in the region C-terminal to Tyr(P) are boxed. The mutation sites (Gly<sup>129</sup> and Arg<sup>147</sup>) are indicated by vertical arrows. The nonconserved Arg<sup>151</sup> is underlined.

kinase activity (17). Substantial evidence supports a role for CHK as a negative growth regulator of human breast cancer through inhibition of ErbB-2/neu-mediated Src family kinase activity. Overexpression of CHK in MCF-7 breast cancer cells markedly inhibits the cell growth, transformation, and invasion induced by heregulin and also causes a significant delay of cell entry into mitosis. Furthermore, the tumor growth of wild-type CHK-transfected MCF-7 cells in nude mice is significantly inhibited compared with that of non-transfected MCF-7 cells or cells transfected with kinase-dead CHK (18). The specific expression of CHK in breast cancer tissues and its inhibitory effect on cancer development strongly suggest the potential of the CHK protein as an anticancer drug and a target of gene therapy.

Mechanism-based target identification and structure-based drug design are promising for the development of selective anticancer drugs that would replace conventional cancer chemotherapy and its associated cytotoxic side effects (19). Precise biochemical and structural information on the CHK SH2 domain and the Tyr(P)<sup>1248</sup> containing peptide is necessary to develop CHK as a potential target of breast cancer therapy. We compared the primary sequence of the CHK SH2 domain with those of other SH2 domains (Fig. 1). The structures of a number of other SH2 domains and their complexes with phosphopeptides derived from biological targets were studied by NMR and x-ray crystallography. All contain similar secondary structural elements (Fig. 1) (20–22). The structures reveal general Tyr(P)-binding sites as well as specificity-determining sites in the SH2 domains. Although the mode of recognition of the cognate phosphopeptides by two types of SH2 domains is different (see "Discussion"), the geometry of the Tyr(P)-binding pocket within each SH2 domain is conserved in the SH2 domain family (23). SH2 domains display positively charged pockets lined with consensus basic residues of Arg (αA1), Arg (βB5), His (βD4), and Lys/Arg (βD6) (Fig. 1). Extensive interactions have been identified between the sequence C-terminal to Tyr(P) of the peptide and several residues in the βD strand, the βD-αB loop, and the αB-βE loop, which play a regulatory role in the specific binding. We constructed three CHK SH2 domain binding mutants in αA and βB to test the biological significance of their binding in growth inhibitory function. We also per-

formed NMR experiments to identify, in the CHK SH2 domain, the binding sites for a phosphotyrosine-containing peptide derived from ErbB-2.

CHK has been suggested to have a specific role in breast cancer and to be a potential target for breast cancer drug development. Mutation of residues to confer modified binding to Tyr(P)<sup>1248</sup> of ErbB-2/neu will elicit functional insights into the binding of CHK to this receptor.

#### EXPERIMENTAL PROCEDURES

**Materials**—Recombinant heregulin-β1 (amino acids 177–244) was obtained from Genentech, Inc. (San Francisco, CA). Anti-phospho-HER2/ErbB-2 (Tyr<sup>1248</sup>) antibody was purchased from Cell Signaling Technology, Inc. The primers for PCR were purchased from Integrated DNA Tech. ECL reagents were purchased from Amersham Biosciences.

**Cell Lines**—Three different breast cancer cell lines with various levels of ErbB-2/neu protein expression were used: MCF-7 (normal level expression), T47D (moderate level overexpression), and BT474 (high level overexpression). All three cell lines were obtained from American Type Culture Collection (Manassas, VA). Cells were grown in RPMI 1640 medium (Cellgro, Inc.) supplemented with 10% fetal bovine serum and 3.5 μg/ml insulin (Sigma). Prior to stimulation with heregulin, cells were starved overnight in medium containing 1% fetal bovine serum and then incubated for 4 h in serum-free medium.

**Peptide Synthesis and Purification**—A peptide containing Tyr(P)<sup>1248</sup> of ErbB-2, ENPEpYLGLDV, was synthesized using solid-phase Fmoc (N-(9-fluorenyl)methoxycarbonyl)-based peptide synthesis with an acetylated N terminus and amidated C terminus (Tufts Core Facility, Boston, MA). All peptides were purified by C<sub>18</sub> reverse-phase high performance liquid chromatography, and identities were confirmed using matrix-assisted laser desorption/ionization mass spectroscopy.

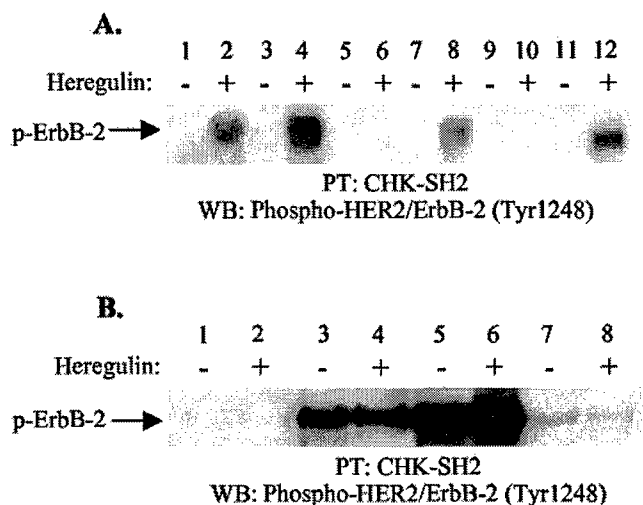
**Construction and Purification of the CHK SH2 Domain**—The CHK SH2 domain constructs (residues 116–217: G129R, R147A, R147K, and wild-type) were subcloned into the pGEX2T vector using the restriction endonuclease sites *Bam*HI and *Eco*RI. Point mutations were generated by PCR using the QuikChange site-directed mutagenesis system (Stratagene) according to the manufacturer's instructions. Mutants were verified by sequencing. At least three independently generated mutants were tested for each construct. The glutathione *S*-transferase (GST)-fused CHK SH2 domains were expressed in bacteria (BL21(DE3) cells) and purified following published procedures (13, 14). Isolated SH2 domains were generated by thrombin cleavage, followed by purification on a benzamidine-Sepharose 6B column (Amersham Biosciences) (13, 14). Approximately 10 mg of protein from all constructs were purified from 1-liter cultures in rich medium (LB medium) and 5 mg from culture in minimal medium.

**Generation of CHK-encoding pIRES2-EGFP Vectors**—To investigate the effects of the generated mutants in breast cancer cells, the same mutations were generated in the full-length form of the CHK gene originating from previously described pcDNA3-based constructs (19–21). The generation and characterization of a CHK mutant lacking kinase activity were described in detail previously (15). All studied forms of the CHK gene were cloned into the pIRES2-EGFP mammalian expression vector (CLONTECH). Expression of wild-type as well as mutant CHK proteins was assessed by transient transfection of 293T cells and by Western blot analysis.

**Binding of ErbB-2 to GST Fusion Proteins**—T47D or BT474 cells ( $\sim 5 \times 10^6$  cells/plate) were starved overnight in medium containing 1% fetal bovine serum, followed by additional starvation in serum-free medium for 4 h at 37 °C. The starved T47D or BT474 cells were then stimulated with 20 nM heregulin for 8 min at room temperature. The stimulation was terminated by the addition of ice-cold lysis buffer (0.1% SDS and 1% Triton X-100 in Tris-buffered saline containing 10% glycerol, 1 mM EDTA, 0.5 mM  $\text{Na}_3\text{VO}_4$ , and protease inhibitor mixture (Roche Molecular Biochemicals)). Lysates were precleared by centrifugation (14,000 rpm, 15 min) and incubated for 90 min at 4 °C with 10  $\mu\text{g}$  of GST fusion proteins coupled to glutathione-Sepharose beads. Next, the beads were washed three times with lysis buffer. SDS buffer was then added, and samples were analyzed on an SDS-7% polyacrylamide gel. Proteins were transferred onto Immobilon-PM membranes (Millipore Corp.), and bound proteins were immunoblotted with anti-phospho-HER2/ErbB-2 (Tyr<sup>1248</sup>) antibody. The blots were developed using the ECL system.

**NMR Spectroscopy**— $^{15}\text{N}$ ,  $^{13}\text{C}$ -Double-labeled protein samples or  $^{15}\text{N}$ -labeled samples were obtained by growing the transformed bacteria in minimal medium containing  $^{15}\text{NH}_4\text{Cl}$  and  $^{13}\text{C}$ -labeled glucose or  $^{15}\text{NH}_4\text{Cl}$  and unlabeled glucose as the sole sources of nitrogen and carbon, respectively (22–24). Protein was purified following the same procedures as described above, except that the purified proteins were concentrated using Centricon centrifugation filtration units (Millipore Corp.) with a  $M_r$  3000 cutoff. The purified proteins were then exchanged into the final NMR sample buffer containing 50 mM phosphate (pH 7.5), 50 mM NaCl, 1 mM EDTA, and 2 mM perdeuterated dithiothreitol (Cambridge Isotope Laboratories, Cambridge, MA). Optimal conditions were predetermined using microdialysis against a variety of buffers (25–27). NMR experiments were performed on Bruker AMX 500-MHz and Avance 600-MHz spectrometers. Titration of the protein with the dissolved peptide (in the same buffer) was monitored by changes in  $^{15}\text{N}$ - $^1\text{H}$  heteronuclear single quantum correlation (HSQC) spectra collected at peptide/protein molar ratios of 0, 0.25, 0.5, 0.75, 0.85, 1.0, 1.25, 1.5, and 2.0. Dissociation constants ( $K_d$ ) of peptide binding were determined by analyzing the titration data assuming fast exchange and by using CRVIT (a nonlinear least-squares fitting program obtained from R. Boyko and B. D. Sykes). For backbone assignment of the protein, triple resonance experiments, including HNCA, HN(CO)CA, HNCACB, and HN(CO)CACB (27, 28), were performed and  $^{15}\text{N}$ -separated three-dimensional nuclear Overhauser effect correlation and total correlation spectra were recorded. The data were processed and analyzed using FELIX 98 (Accelrys, Inc.).

**In Vitro Src Tyrosine Kinase Assay**—For this experiment, MCF-7 cells were chosen because of their overexpression of Src kinase. The cells were grown on 100-mm Petri dishes until 80% confluent and then transfected with 10  $\mu\text{g}$  of various CHK-pIRES2-EGFP constructs using LipofectAMINE 2000 (Invitrogen) as recommended by the manufacturer. Twenty-four hours after transfection, the cells were starved overnight in medium containing 1% fetal bovine serum, followed by additional starvation in serum-free medium for 4 h at 37 °C. The starved MCF-7 cells were then stimulated with 20 nM heregulin for 8 min at room temperature, and total protein extracts were prepared as described above. One milligram of protein was immunoprecipitated using antibodies against Src (Santa Cruz Biotechnology). The immunoprecipitates were washed three times with lysis buffer and then resuspended in 30  $\mu\text{l}$  of kinase buffer (50 mM Tris-HCl (pH 7.4), 10 mM  $\text{MnCl}_2$ , 10 mM  $\text{MgCl}_2$ , 0.1% Triton X-100, and 1 mM dithiothreitol) containing phosphatase and protease inhibitors, 0.25 mg/ml poly(Glu/Tyr) (4:1; Sigma) as an exogenous kinase substrate, 10  $\mu\text{M}$  unlabeled ATP, and 10  $\mu\text{Ci}$  of [ $\gamma$ - $^{32}\text{P}$ ]ATP (6000 Ci/mmol; PerkinElmer Life Sciences). After 10 min at 30 °C, the reaction was stopped by adding SDS sample buffer and boiling the samples for 10 min. Subsequently, the samples were resolved on SDS-12% polyacrylamide gels, and the gels were stained with Coomassie Blue. The labeled poly(Glu/Tyr) was excised from the gel, and radioactivity was counted.



**FIG. 2. GST pull-down experiment.** Serum-starved T47D (A) or BT474 (B) cells were stimulated with 20 nM heregulin for 8 min at room temperature and then lysed in protein lysis buffer. Lysates were incubated with GST fusion proteins coupled to glutathione-Sepharose beads. The precipitated proteins were developed on an SDS-7% polyacrylamide gel and then transferred onto Immobilon-PM membranes. Bound proteins were immunoblotted with anti-phospho-HER2/ErbB-2 (Tyr<sup>1248</sup>) antibody. A: lanes 1 and 2, wild-type CHK SH2 domain; lanes 3 and 4, G129R; lanes 5 and 6, R147A; lanes 7 and 8, R147K; lanes 9 and 10, empty GST beads; lanes 11 and 12, total cell lysate. B: lanes 1 and 2, empty GST beads; lanes 3 and 4, wild-type CHK SH2 domain; lanes 5 and 6, G129R; lanes 7 and 8, R147A. PT, precipitation; WB, Western blot.

## RESULTS

### Generation of the Binding Mutants

Two arginine residues are conserved in the Tyr(P)-binding pocket. Alignment of the CHK SH2 domain with other SH2 domain sequences shows that whereas the arginine in  $\beta\text{B5}$  is present in these SH2 sequences, the  $\alpha\text{A2}$  CHK SH2 domain has a glycine. This arginine in the binding pocket of other SH2 domains provides a positive charge to coordinate the phosphate of Tyr(P), implying either a different mode of binding or weak binding for CHK. Arg<sup>147</sup> in  $\beta\text{B}$  is a critical residue for Tyr(P) binding and is strictly conserved in SH2 domains (23). Based on these observations, we constructed three CHK SH2 domain mutants: G129R, R147A, and R147K. The G129R mutant encodes a CHK SH2 domain protein in which the  $\alpha\text{A2}$  glycine at the phosphotyrosine-binding pocket has been replaced by arginine. For the R147A and R147K mutants, the  $\beta\text{B5}$  arginine has been replaced by alanine and lysine, respectively. Our hypothesis is that G129R will have enhanced ErbB-2 phosphopeptide binding, R147A will have disrupted binding, and R147K will have reduced binding.

### Binding Studies

**GST Pull-down Experiment**—We conducted binding studies with the three CHK SH2 domain mutants as well as with wild-type CHK (Fig. 2). Two different breast cancer cell lines, T47D (moderate level of ErbB-2/neu expression) and BT474 (high level of expression), were tested. The conserved tyrosine residues of overexpressed ErbB-2/neu in BT474 cells were found to be autophosphorylated in the absence of ligand stimulation. The cells were serum-starved as described under “Experimental Procedures” and then activated with heregulin (20 nM) for 8 min. Unstimulated and stimulated cells were lysed and precipitated with GST-CHK SH2 fusion proteins as well as with GST protein alone. The precipitates were analyzed by SDS-PAGE and immunoblotted with anti-phospho-HER2/ErbB-2 (Tyr<sup>1248</sup>) antibody. In comparison with wild-type CHK,

the substitution of Gly<sup>129</sup> with Arg<sup>129</sup> resulted in dramatically increased binding in both cell lines. In T47D cells (Fig. 2A), only heregulin stimulation induced the association of ErbB-2 with the purified wild-type SH2 domain and also G129R, indicating that G129R binding is ligand-stimulated to a similar level compared with wild-type binding. The substitution of Arg<sup>147</sup> with Lys<sup>147</sup> slightly decreased binding, whereas the substitution with Ala<sup>147</sup> completely disrupted binding. These results indicate that the positive charge of Arg<sup>147</sup> is critical for phosphopeptide binding and that the length of the side chain has a moderate effect on binding. Because the R147K mutant showed only a moderate effect on binding in comparison with the wild-type SH2 domain, the R147K mutant was not analyzed in further experiments. In BT474 cells, no ligand stimulation was necessary for the CHK SH2 domain interaction with the constitutively phosphorylated ErbB-2/neu protein (Fig. 2B). Again, almost no association of R147A with ErbB-2/neu was seen, whereas G129R pulled down markedly more ErbB-2 protein than did the wild-type CHK SH2 domain.

**NMR Experiments**—The backbone atoms of the CHK SH2 domain were assigned using triple resonance experiments (Fig. 3A). NMR, which is a method used to determine the high resolution structure of macromolecules, is particularly valuable in providing rapid identification of a ligand-binding site. The <sup>15</sup>N-<sup>1</sup>H HSQC experiment yields a well resolved spectrum, with single peaks for the backbone amides of most residues in the protein (25). Changes in the positions of these peaks upon titration of the ligand can identify the residues in the binding site, and analysis of the titration data can be used to determine the kinetics of binding (24).

To analyze the interaction between CHK SH2 domain constructs and the phosphopeptide, we titrated the peptide into 0.6 mM <sup>15</sup>N-labeled SH2 domains. Progressive changes in <sup>1</sup>H and <sup>15</sup>N chemical shifts were monitored with a series of <sup>15</sup>N HSQC spectra. Significant chemical shift changes in the wild-type SH2 domain were observed for several residues in the αA, βB, and βD secondary structures as well as in the βD-αB and αB-βE loops (Fig. 3A), which have been implicated in the binding of other SH2 domains to phosphopeptides (26). In particular, several positive residues in βD (His<sup>168</sup> and Arg<sup>170</sup>) undergo large chemical shift changes upon complex formation, consistent with the positive charged residues in these secondary structural elements forming contacts with Tyr(P) of the ligand. In the αA helix, several residues changed chemical shifts upon complex formation (Ile<sup>127</sup>, Gly<sup>129</sup>, Gln<sup>134</sup>, and Gln<sup>135</sup>). The βB-βC loop has also been implicated in the binding of the peptide, and significant chemical shift changes were observed for residues in this loop (Ser<sup>149</sup>, Arg<sup>151</sup>, and Gly<sup>154</sup>). Interestingly, Arg<sup>147</sup> showed little change in chemical shift despite its presumed role in contacting the phosphate of the ligand. Significant chemical shift changes were also observed in residues in βD (Tyr<sup>169</sup>, Val<sup>171</sup>, and Leu<sup>172</sup>), the βD-αB loop (Ile<sup>180</sup> and Asp<sup>181</sup>), and the αB-βE loop (Ile<sup>203</sup>), which presumably form the hydrophobic environment for the hydrophobic residues of the C terminus to the Tyr(P) region of the peptide (see Fig. 5).

G129R, which showed enhanced binding, was also titrated with the phosphopeptide (Fig. 3B). The Gly<sup>129</sup> resonance was replaced with a new resonance (Arg<sup>129</sup>), consistent with the substitution of glycine with arginine and the expected changes in chemical shift (27). Several residues showed chemical shift changes upon mutation. In particular, residues in αA (Ile<sup>127</sup>, Glu<sup>131</sup>, Ala<sup>132</sup>, and Gln<sup>134</sup>) experienced chemical shift changes (0.3 ± 0.1 ppm) presumably due to introduction of the long charged side chain of Arg. Several residues in βB and βD also showed chemical shift changes, suggesting that they are in proximity to the G129R mutation site. Upon titration with

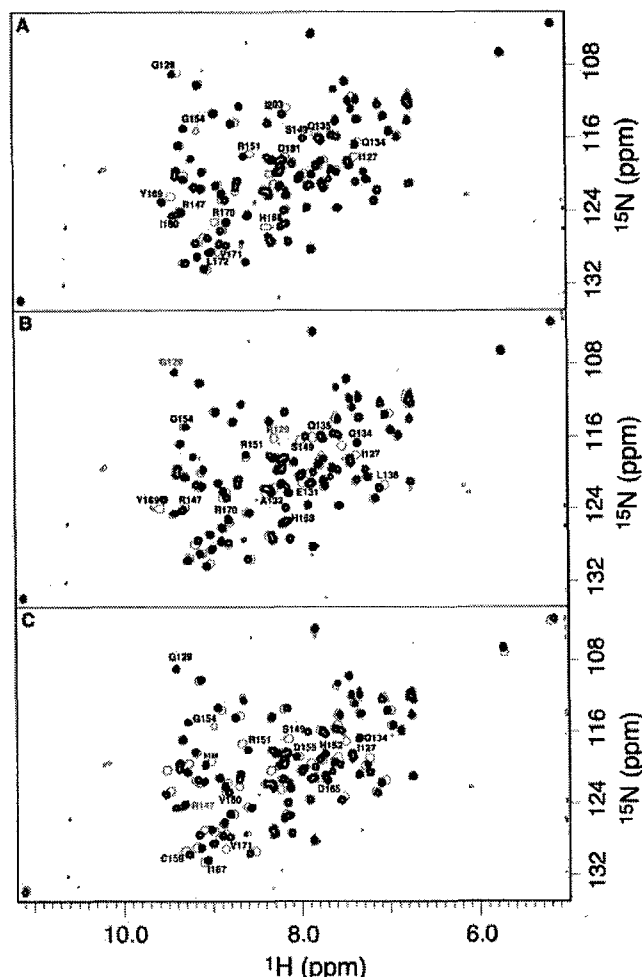


FIG. 3. A, superimposed <sup>15</sup>N-<sup>1</sup>H HSQC spectra of the wild-type CHK SH2 domain without (black) and with (red) 1 eq of the Tyr(P) peptide (Ac-ENPEpYLGLDV-NH<sub>2</sub>). B, superimposed <sup>15</sup>N-<sup>1</sup>H HSQC spectra of the wild-type CHK SH2 domain (black) and the G129R mutant (red). C, superimposed <sup>15</sup>N-<sup>1</sup>H HSQC spectra of the wild-type CHK SH2 domain (black) and the R147A mutant (red). The 500-MHz spectra were collected at 25 °C from samples containing ~0.6 mM SH2 domain. Peaks that significantly changed chemical shifts upon binding the peptide (A) or upon mutation (B and C) are labeled in black by residue number. Residues targeted for mutation are labeled in red.

peptide, residues similar to the wild-type residues underwent significant chemical shift changes, except for residues near the mutation site in αA and βB (Table I).

R147A did not show chemical shift changes with any residue upon peptide titration, consistent with the complete disruption of binding seen in the GST pull-down experiment. With this mutation, the peak for Arg<sup>147</sup> disappeared, but a new Ala<sup>147</sup> peak was difficult to identify, presumably due to resonance overlap (Fig. 3C). Compared with G129R, the R147A substitution showed different chemical shifts for residues in regions such as αA (Ile<sup>127</sup>, Ser<sup>128</sup>, and Gly<sup>129</sup>), βB (Ser<sup>149</sup> and His<sup>152</sup>), the βB-βC loop (Gly<sup>154</sup> and Asp<sup>155</sup>), βC (Cys<sup>159</sup> and Val<sup>160</sup>), and βD (Asp<sup>165</sup>, Ile<sup>167</sup>, and Val<sup>171</sup>). In addition, Gly<sup>129</sup> disappeared with the mutation, implying line broadening consistent with the introduction of a motion on the millisecond-to-microsecond time scale. Although both mutants showed perturbation at several residues, the overall structure seemed to be unaltered, as the majority of the residues showed little change (Fig. 3).

Equilibrium dissociation constants (*K<sub>d</sub>*) were determined from a plot of chemical shift changes versus the ratio of peptide to protein. Chemical shifts of residues Ile<sup>127</sup> (βA-αA loop);

TABLE I  
Equilibrium dissociation constants and chemical shift changes of the wild-type SH2 domain and G129R

Residue <sup>a</sup>	Wild-type SH2		G129R	
	<i>K<sub>d</sub></i>	Shift <sup>b</sup>	<i>K<sub>d</sub></i>	Shift <sup>b</sup>
	mM	ppm	mM	ppm
Ile <sup>127</sup>	ND	ND	0.41 ± 0.27	0.19 ± 0.04
Gly <sup>129</sup>	1.1 ± 0.7	0.18 ± 0.06	ND	ND
Arg <sup>147</sup>	ND	ND	0.68 ± 0.50	0.09 ± 0.03
Gly <sup>154</sup>	0.74 ± 0.28	0.52 ± 0.09	0.09 ± 0.03	0.68 ± 0.50
Tyr <sup>156</sup>	0.63 ± 0.14	0.33 ± 0.03	0.05 ± 0.05	0.20 ± 0.03
His <sup>168</sup>	0.29 ± 0.04	0.62 ± 0.03	ND	ND
Tyr <sup>169</sup>	0.27 ± 0.07	0.42 ± 0.03	0.27 ± 0.07	0.42 ± 0.03
Arg <sup>170</sup>	0.23 ± 0.05	0.25 ± 0.01	0.26 ± 0.10	0.20 ± 0.02
Ile <sup>203</sup>	1.89 ± 0.96	0.68 ± 0.22	0.15 ± 0.17	0.20 ± 0.03
Average	0.48 ± 0.05	ND	0.13 ± 0.08	ND

<sup>a</sup> These residues were selected because they were well resolved during peptide titration. ND, not determined because chemical shift differences were too small (Ile<sup>127</sup>, Gly<sup>129</sup>, and Arg<sup>147</sup>) or because of spectral overlap (His<sup>168</sup>).

<sup>b</sup> The chemical shift difference was calculated from the absolute value of the change in <sup>1</sup>H chemical shift plus 0.2 times the absolute value of the change in <sup>15</sup>N chemical shift.

Gly<sup>129</sup> (αA); Arg<sup>147</sup> (βB); Gly<sup>154</sup> (βB-βC loop); Tyr<sup>156</sup> (βC); His<sup>168</sup>, Tyr<sup>169</sup>, and Arg<sup>170</sup> (βD); and Ile<sup>203</sup> (αB-βE loop) were monitored because these residues were involved in the phosphopeptide interaction and remained well resolved throughout the titration. Consistent with the results from the GST pull-down binding assay, overall, wild-type CHK has a *K<sub>d</sub>* of ~0.5 mM, showing five times weaker affinity than G129R (Table I). In wild-type CHK, residues in βD (His<sup>168</sup>, Tyr<sup>169</sup>, and Arg<sup>170</sup>) showed tighter binding than Gly<sup>154</sup>, Tyr<sup>156</sup>, and Ile<sup>203</sup>, which are predicted to be the C-terminal residues of the bound ErbB-2 phosphopeptide. In G129R, the affinity of Gly<sup>154</sup>, Tyr<sup>156</sup>, and Ile<sup>203</sup> increased >10 times over that seen in wild-type CHK, whereas the residues in βD (His<sup>168</sup>, Tyr<sup>169</sup>, and Arg<sup>170</sup>) remained the same as observed in wild-type CHK.

#### Src Kinase Assay

To investigate the functional role of the association of CHK and ErbB-2, we tested the effects of transient transfection with CHK-pIRES2-EGFP constructs on heregulin-stimulated Src kinase activity in MCF-7 cells. The expression levels of all studied forms of the CHK protein were similar as assessed by Western blotting (Fig. 4). As shown in Fig. 4, stimulation of MCF-7 cells with heregulin caused a dramatic increase in the activity of Src kinase. Transfection with the wild-type CHK gene strongly inhibited the heregulin-stimulated Src activity, whereas no significant difference was seen in cells transfected with the kinase-dead mutant of CHK or an empty vector control. Furthermore, the inhibition of heregulin-stimulated Src kinase activity was markedly diminished by R147A, whereas the G129R-mediated inhibition was somewhat stronger compared with wild-type CHK.

#### DISCUSSION

The interaction of SH2 domains and their phosphotyrosine-containing binding partners has been extensively studied because of its critical role in signal transduction and cancer development (23, 28). Studying the binding of the CHK SH2 domain to the ErbB-2 receptor is particularly important for understanding the development of breast cancer (17). Studies using phosphopeptide libraries to probe sequence specificity showed that there are two types of SH2 domains, types I and II (23). Type I SH2 domains involve non-receptor tyrosine kinases such as Src, Lck, Fyn, and Abl and exhibit preferences for the motif Tyr(P)-hydrophilic-hydrophilic-(Ile/Pro) (29). Type II SH2 domains include phospholipase Cγ1, Syp tyrosine phosphatase,

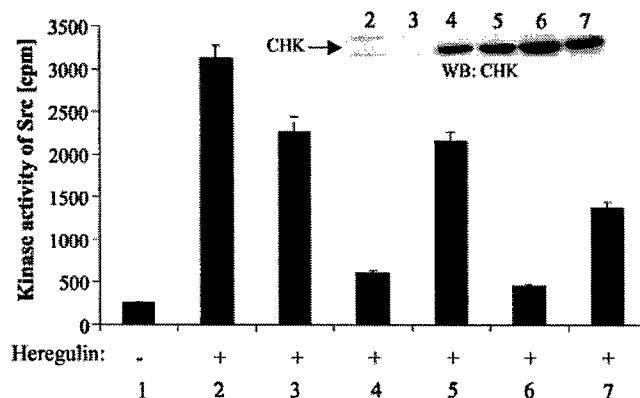


FIG. 4. **Src kinase assay.** MCF-7 cells were transiently transfected with 10 μg of various forms of CHK. The serum-starved cells were then stimulated with 20 nM heregulin for 8 min at room temperature, and total protein extracts were prepared. One milligram of protein was immunoprecipitated using antibodies against Src. The tyrosine kinase assay was performed as described under "Experimental Procedures." Lanes/bars 1 and 2, non-transfected MCF-7 cells; lanes/bars 3, empty vector; lanes/bars 4, wild-type CHK; lanes/bars 5, kinase-dead CHK; lanes/bars 6, G129R; lanes/bars 7, R147A. Inset, expression of various CHK proteins in transfected cells was assessed by Western blot (WB) analysis.

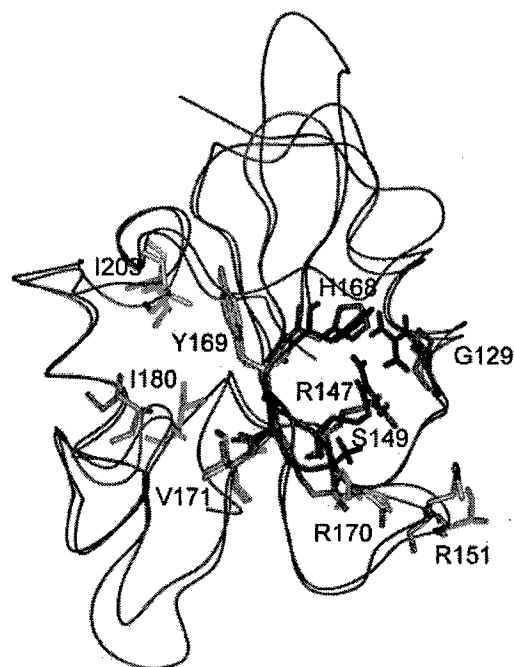


FIG. 5. **Superposition of the phosphopeptide-binding site of the CHK SH2 domain (red; Protein Data Bank code 1JWO) and the v-Src SH2 domain (blue; Protein Data Bank code 1BKL).** Residues involved in binding Tyr(P) (Gly<sup>129</sup>, Arg<sup>147</sup>, His<sup>168</sup>, and Arg<sup>170</sup>) are indicated in red for CHK and in blue for the corresponding residues of Src. Residues composing the hydrophobic site that interact with residues composing the C terminus of the Tyr(P) region of the phosphopeptide are indicated in pink (CHK) and in green (Src). The nonconserved Arg<sup>151</sup> of CHK is indicated in pink.

Shc, and Grb2 and prefer the motif Tyr(P)-hydrophobic-X-hydrophobic. Structural analysis of the type I high affinity binding ( $10^{-8}$  M) SH2 domain-peptide complex showed that Tyr(P) (in pYEEI) is inserted into a large positively charged pocket and Ile into a smaller hydrophobic pocket. In contrast, the type II phospholipase Cγ1-peptide complex has relatively lower affinity binding ( $K_d = 10^{-6}$  M) and demonstrates more extensive peptide interactions, using a long hydrophobic groove to accommodate the two hydrophobic residues in the peptide.

The CHK SH2 domain binds to the ErbB-2 phosphopeptide (pYLGLDV), which contains the consensus sequence for binding to a type II SH2 domain (30). HSQC experiments identified the residues involved in phosphopeptide binding. Residues of the positively charged Tyr(P)-binding site of CHK, as observed in other SH2 domain-peptide interactions, are predicted to be Gly<sup>129</sup> in  $\alpha$ A, Arg<sup>147</sup> in  $\beta$ B, and His<sup>168</sup> and Arg<sup>170</sup> in  $\beta$ D. Residues of this binding site participate in electrostatic interactions with the Tyr(P) ring. The hydrophobic residues in the specificity-determining site ( $\beta$ D,  $\beta$ D- $\alpha$ B loop, and  $\alpha$ B- $\beta$ E loop) provide extensive hydrophobic contacts between the C-terminal hydrophobic residues and Tyr(P) of the peptide. Recently, the crystal structure of the CHK SH2 domain has been solved by Murthy *et al.* (34) (Protein Data Bank code 1JWO). The overall folding of the CHK SH2 domain is similar to that of other SH2 domains. Superposition of backbone atoms of the secondary structure residues of the CHK and Src SH2 domains showed that the overall folding and binding pocket are conserved (Fig. 5). The side chain of the nonconserved Arg<sup>151</sup> of the CHK SH2 domain is positioned near the Tyr(P)-binding site. The positive charge of this residue may also contribute to binding. The chemical shift of Arg<sup>151</sup> was changed by peptide addition.

The dissociation constant of the wild-type CHK SH2 domain was found to be 0.5 mM, which is much weaker than that of the low affinity type II SH2 domain (23). The low affinity for the phosphopeptide does not necessarily mean a low affinity of ErbB-2/neu for CHK *in vivo* in cells. Because Tyr(P)<sup>1248</sup> is the most critical residue in ErbB-2/neu-mediated cancer development, dynamic regulation can be involved in its interaction. We suspect that the unusual presence of Gly<sup>129</sup> in the Tyr(P)-binding site is one reason for this weak binding. The GST pull-down experiment showed a dramatic enhancement of the binding when Gly<sup>129</sup> was substituted with Arg<sup>129</sup>. The  $K_d$  of G129R decreased by >5-fold, as determined from the NMR titration experiments. Both experiments indicated that G129R showed higher binding affinity for the ErbB-2 peptide. The similar chemical shift changes of the residues in the specificity-determining site of G129R and the wild-type SH2 domain indicate no alteration in specificity. Interestingly, the increased affinity of G129R is caused by a dramatic increase in the binding affinity of residues that are likely to bind to the C-terminal residues of the ErbB-2 phosphopeptide (Table I).

The R147K mutation decreased binding, whereas the R147A mutation completely disrupted binding. This result implies that the positive charge of Arg<sup>147</sup> is critical to coordinate the negative charge of Tyr(P) and that the length of the side chain also affects the binding. The importance of Arg<sup>147</sup> in SH2 domains has been tested in different systems, and the results indicate that this residue is critical for binding (31, 32). Alanine mutation of this strictly conserved Arg<sup>147</sup> ( $\beta$ B) resulted in a large increase in  $\Delta G_0$  ( $\Delta\Delta G_0 = 3.2$  kcal/mol), whereas mutations of other residues each resulted in a significantly smaller ( $\Delta\Delta G_0 < 1.4$  kcal/mol) reduction in affinity. This indicates that Arg<sup>147</sup> ( $\beta$ B) is an important determinant of the Tyr(P)-binding recognition site (33).

How can CHK be involved in negative growth regulation in human breast cancer? How can the specific binding of CHK to the most critical and strictly conserved autophosphorylation site (Tyr(P)<sup>1248</sup>) of ErbB-2 be related to the down-regulation of ErbB-2-mediated Src family kinase activity? One model is that upon heregulin stimulation, CHK association with the ErbB-2 receptor locates the CHK near the substrate, Src, thereby causing growth inhibitory effects (13, 14). The enhanced binding or disrupted binding mutants were used to test whether the specific association of CHK and ErbB-2 is related to Src kinase

activity. The R147A mutant markedly diminished the inhibition of Src activity, whereas the G129R mutant inhibited Src activity more potently in comparison with the wild-type SH2 domain. These results prove that the specific interaction of CHK and ErbB-2 via the SH2 domain is directly related to the growth inhibitory effects of CHK.

Because CHK shows restricted expression and specifically inhibits breast cancer development, CHK can be a potential candidate for gene therapy. However, the expression levels of CHK are very low in breast tumors, and CHK barely shows kinase activity in breast cancer tissues as compared with Csk (13–16). Because highly effective inhibition of Src activity is important for gene therapy, improved expression of the CHK gene, development of CHK with higher kinase activity, and construction of a higher affinity CHK SH2 domain would be very important for inhibiting breast cancer growth. Thus, the enhanced binding mutant (G129R) is promising as a potential candidate for gene therapy.

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**Csk-homologous kinase (CHK) – the role in mammary development and tumorigenesis in transgenic mouse model.**

Radoslaw Zagozdzon<sup>1</sup>, Rafal Kaminski<sup>1,2</sup>, Yigong Fu<sup>1</sup>, Pawel Mroz<sup>1,2</sup>, Wei Fu<sup>1</sup>, Hava K. Avraham<sup>1,#</sup>

<sup>1</sup> Division of Experimental Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, 4 Blackfan Circle, Boston, MA 02115

<sup>2</sup> Current addresses: [R.K.] Department of Immunology, Center of Biostructure, Medical University of Warsaw, ul. Chalubinskiego 4, 02-446 Warsaw, Poland; [rkaminski@ib.amwaw.edu.pl](mailto:rkaminski@ib.amwaw.edu.pl); [P.M.] Wellman Laboratory of Photomedicine, Department of Dermatology, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114

# Correspondence to: Hava Avraham, Beth Israel Deaconess Medical Center, Division of Experimental Medicine, 3<sup>rd</sup> floor, HIM bldg., 4 Blackfan Circle, Boston, MA 02115; email: [havraham@bidmc.harvard.edu](mailto:havraham@bidmc.harvard.edu)

Running title: CHK in mammary carcinogenesis.

## **Abstract.**

An essential step in the initiation of the mitogenic response to HER2/Neu activation is caused by the activation of  $\alpha$ -Src or other Src family kinases (SFKs). Most of the data supporting this notion originate, however, from in vitro studies. To validate the necessity of Src activation for Neu-induced formation of breast cancer, we have decided to use CHK (a natural inhibitor of Src activity) and to generate transgenic mice expressing CHK under MMTV promoter. Overexpression of CHK in mammary tissue did not significantly altered development or function of mammary tissue as assessed by histological studies. MMTV/CHK mice were crossbred with MMTV/Neu and MMTV/NeuT mice. Overexpression of CHK along with Neu significantly inhibited Neu-induced Src in developing mammary tumors. However, in our experiments we did not observed any significant influence of CHK expression on the dynamics of tumor occurrence or growth. We have not also observed a significant effects of CHK on the Neu-induced transformation of MCF10A when studied in the 3D culture in vitro model. Taken together, our results show that downregulation of SFKs activity using CHK overexpression have no significant impact on ErbB2/Neu mediated oncogenesis. This observation warrants further investigation of SFKs role in breast cancer, employing other systems to downregulate the SFKs activity in vivo.

## **Introduction.**

Breast cancer is the second leading cause of cancer death among women in the United States and is the leading cause of death among women aged 30 to 70 (Schultz & Weber, 1999). The onset of 90% of all breast cancers is random and spontaneous, while 10% of cancers have been linked to specific mutations in autosomal dominant breast cancer susceptibility genes such as BRCA1 and BRCA2 (Borg et al., 1991; Claus et al., 1991; Newman et al., 1988). The majority of breast carcinomas appear to be sporadic and to have a complex accumulation of molecular and cellular abnormalities that constitute the malignant phenotype. The random onset of breast cancers has in many cases been correlated with increased HER2/Neu (also termed ErbB2) expression and Src family tyrosine kinase activity (Bishop, 1995; Menard et al., 2000; Muthuswamy et al., 1994; Yamamoto et al., 1986). Overexpression of HER2/Neu in primary breast carcinomas correlates with a poor prognosis for this disease (Rowse et al., 1998). To date no ligands binding directly only HER2/Neu have been identified. However, heterodimerization of HER2/Neu with ErbB1 or ErbB3 (other members of EGF receptor family) could be triggered by EGF or heregulin (Sliwkowski et al., 1994; Wada et al., 1990). Once the HER2/Neu is activated, it becomes autophosphorylated on five tyrosine residues. Although, the individual targets and downstream effectors of each phosphotyrosine are not clear (Dankort et al., 2001), phosphorylated sites act as a docking sites for several proteins: Shc, GAP, PLC?, PI3-K, c-Src (Fazioli et al., 1991; Muthuswamy & Muller, 1995; Peles et al., 1992; Xie et al., 1995).

An essential step in the initiation of the mitogenic response to HER2/Neu activation is caused by the activation of c-Src or other Src family kinases (SFKs) (Vadlamudi et al., 2003). Human breast cancers often show much higher levels of Src protein kinase activity than normal adjacent epithelium (Biscardi et al., 1998; Ottenhoff-Kalff et al., 1992; Verbeek et al., 1996). Activation of Src kinases was assigned as an essential step in mitogenic cascade activation by several receptor tyrosine kinases (Thomas & Brugge, 1997). Involvement of c-Src in two major signaling pathways in human breast cancer has been demonstrated. In human breast carcinoma cell lines, the SH2 domain of Src binds to activated ErbB family receptors: epidermal growth factor



receptor (EGF-R, ErbB1) and HER2/Neu (ErbB2) (Luttrell et al., 1994). c-Src family kinases are activated in ErbB2-induced mammary tumors and this, elevated activity correlates with ability of c-Src kinases capacity to physically associate with ErbB2 (Muthuswamy & Muller, 1995; Muthuswamy et al., 1994). In addition, it has been reported that that c-Src kinases are involved in cellular transformation induced by growth factor stimulation (Thomas & Brugge, 1997), could lead to attachment-independent growth and invasion (Sheffield, 1998; Zrihan-Licht et al., 2000) and are indispensable for progression from G<sub>2</sub> to mitosis (Roche et al., 1995). Association of c-Src with receptor protein tyrosine kinases is an integral part of the signaling events mediated by the receptors, and may contribute to the malignant transformation of cells (reviewed in (Hynes, 2000)).

Src family kinase activity is inhibited by the phosphorylation of a conserved, carboxy-terminal tyrosine (Cooper & Howell, 1993). The protein tyrosine kinase responsible for this phosphorylation is Csk (Okada & Nakagawa, 1989). We and others identified a second member of the Csk family – Csk Homologous Kinase (CHK), previously referred to as Matk, Ctk, Hyl, Ntk, Lsk, or Batk (Avraham et al., 1995; Bennett et al., 1994; Chow et al., 1994; Davidson et al., 1997; Hamaguchi et al., 1994; Jhun et al., 1995; Klages et al., 1994; Kuo et al., 1994a; McVicar et al., 1994; Sakano et al., 1994). CHK and Csk structurally share 53% amino acid identity overall and 59% amino acid identity within the catalytic domain (Bennett et al., 1994; Chow et al., 1994; Klages et al., 1994; Kuo et al., 1994a; McVicar et al., 1994; Sakano et al., 1994). Like Csk, CHK phosphorylates the inhibitory carboxy-terminal tyrosine of several Src family kinases, including Lck, Fyn, c-Src and Lyn (Avraham et al., 1995; Chow et al., 1994; Davidson et al., 1997; Klages et al., 1994). Unlike Csk, which is ubiquitously expressed, the expression of CHK is limited to neuronal and hematopoietic cells (Bennett et al., 1994; Chow et al., 1994; Klages et al., 1994; Kuo et al., 1994a; McVicar et al., 1994; Sakano et al., 1994).

CHK has been suggested to have a specific role in breast cancer and to be a potential target of breast cancer drug development. CHK has been shown to be specifically expressed in primary breast cancer specimens, but not in normal breast tissues (Bougeret et al., 2001; Zrihan-Licht et al., 1998). CHK was shown to be expressed

in 87.5% of breast carcinomas whereas CHK was not detected by immunofluorescence staining in none of normal breast tissue specimens. Our functional studies in the T47D breast cancer cell line demonstrated a specific interaction between CHK and activated HER2/Neu upon heregulin stimulation (Zrihan-Licht et al., 1997). Our previous studies also showed that upon heregulin stimulation of breast cancer cells there was extensive elevation of Src kinase activity, and that overexpression of CHK completely inhibited this activity (Zrihan-Licht et al., 1998). In addition, overexpression of CHK in MCF-7 breast cancer cells markedly inhibited cell growth and proliferative response to heregulin, as well as decreased colony formation in soft agar (Zrihan-Licht et al., 1998). Therefore, CHK is able to inhibit HER2/Neu-activated src kinases and has the potential to block breast cancer cell growth.

Noteworthy, besides the inhibition of Src activity, reports also exist suggesting some positive role of CHK in tyrosine kinase receptor signaling. In neural cells, CHK was shown to potentiate the activation of MAPK pathway (Kim et al., 2004; Kuo et al., 1997; Yamashita et al., 1999). Also, one of our recent reports (Zagozdzon et al., 2002) shows facilitated activation of PI3-K-Akt pathway in MCF-7 cells overexpressing CHK. Both those pathways (Erk1/2 and Akt-mTor) were recently assigned to the disruption of mammary acinar structure and spreading through reconstructed basal membrane (Debnath et al., 2003b; Seton-Rogers et al., 2004).

All these observations have suggested specific and important role for CHK in breast cancer. However, most studies on the role of CHK in breast cancer were done using histological specimens or cell lines. To confirm these findings, we find imperative to study the effects of CHK on mammary tumor progression in vivo using genetically modified animals. The advent of transgenic mice now permits manipulation of mouse genome with the aim of studying the involvement of particular genes in tumorigenesis and disease progression and of developing mouse models of human genetic disease. The utility of such technologies is emphasized in transgenic mice expressing genes thought to play important roles in the initiation and progression of mammary carcinomas. Many such transgenic mice models have been already generated. In this study, we describe generation of mice expressing CHK in mammary tissue and we present the studies of the role in mammary development and tumorigenesis in transgenic mouse model.

## Results:

Generation of FVB-Tg(MMTV-CHK) mice. From transgenic progeny (F<sub>0</sub>) we obtained 9 animals being positive for transgene in PCR and Southern Blotting (Figure 1B). All positive animals were crossbred to wild type FVB/N mice. All litters were screen for transgene presence using PCR. Positive litters were confirmed for transgene presence using Southern blot (Figure 1C) and used further in single transgenic as well as double transgenic experiments (Figure 1D). Although, 3 strains (#4, #6, #7) gave germ line transition, for further experiments only two of them (#4, #7) were used. Strain #6 show up to be defective, as it gave only birth 1 time and litters died within several weeks. This phenotype was not observed in any other of the strains and it, most probably, could be assigned to transgene insertion site in this strain. As MMTV promoter gives expression also in other tissues than mammary epithelium (Figure 1E and (Muller et al., 1988; Sinn et al., 1987)), detailed microscopic evaluation of several tissues has been performed. We found no significant differences between wild-type animals and MMTV-CHK transgenic mice in any of analyzed tissues (data not shown). Expression of CHK transgene (human isoform) in mammary glands of transgenic mice was confirmed using Western blot (Figure 1F).

### Development of mammary glands in Tg(MMTV-CHK) mice.

Whole mounts were prepared as described under materials and methods section. Glands were examined at several stages of development. Four time-points were chosen: 8 weeks of age adult mice, 16.5 days pregnant mice, lactating mice and glands during involution isolated from mice 3 days after weaning. No alterations were observed in overall patterning of mammary tree at any time point. Our observations suggest, the terminal buds in the Tg(MMTV-CHK) mice are well developed and do not differ from ones in wild-type mammary glands. During progression to pregnancy and lactation we can observe robust growth of mammary tree with progression in number and shape of terminal buds (Figure 2 and data not shown). Noteworthy, these animals do not show apparent abnormalities in feeding fetuses and in microscopic sections taken from

lactating animals we could observe milk in alveoli. No significant differences were observed between MMTV-CHK transgenic strains.

#### Biochemical characterization of mammary glands in Tg(MMTV-CHK) mice.

Protein extracts from mammary glands were analyzed for the presence and/or activity of Src, MAPK, and Akt kinases. The activity of  $\alpha$ -Src kinase was measured using kinase assay (Figure 3A). As expected, the activity of Src was markedly diminished in mammary glands of CHK transgenic mice. Interestingly, another effect of CHK activity, namely the presence of increased amount of phosphorylated p42/p44 MAPK (Erk1/2 mitogen-activated protein kinase) was also found (Figure 3B) in these specimens. On the other hand, the phosphorylated Akt was detected in wild type and transgenic animals at similar levels (data not shown). Most probably, presence of activated form of this kinase in mammary gland is related to its indispensability for the survival of luminal cells in mammary alveoli (Debnath et al., 2002). Those data suggest that overexpressed CHK (as may happen in mammary carcinomas (Zrihan-Licht et al., 1997)) may itself acts as independent activator of mitogen activated pathways. Despite of that, we did not detect hiperplasia or tumors arising in mammary glands of MMTV-CHK transgenic mice during whole period of observation (up to 100 weeks). Similar observations have been made also when CHK was over-expressed in MCF10A cells, cultured in three-dimensional conditions (Figure 4). Mammary cells inlaid onto reconstructed basal membrane created acinis, which did not differ between experimental groups. Average size of formed structures and proliferation rate within acini were similar. We also did not detect any significant differences in apoptosis of cells within acini between different experimental groups.

#### Tumor development in bitransgenic mice carrying single oncogenes as Neu and activated Neu (NeuT) and CHK.

The newly generated animals expressing CHK under mammary tissue-specific promoter were crossbred to MMTV-Neu or MMTV-NeuT (Figure 1D). Double transgenic offsprings were observed for the tumor occurrence for the period of 50-weeks (Figure 5A). Interestingly, despite previous reports of CHK inhibiting the action of ErbB2 in *in*

*vitro* models (Bougeret et al., 2001) in our model we did not detect any significant inhibitory effect of CHK on the tumor appearance in oncogene-expressing mice. Our *ex vivo* experiments showed that there was no differences when number of apoptotic cells (tunnel assay) was counted in tumors arisen in single oncogene expressing mice *versus* those expressing oncogene along with CHK. Similar observation was made when proliferation marker (PCNA) had been assessed by immunohistochemistry in single *versus* bitransgenic animals. Interestingly, as shown on Figure 5B, the activity of c-Src kinase had been barely detectable in tumors derived from double-transgenic mice (expressing an oncogene and CHK in mammary tissue). These experiments indicate that, at least in mouse model, increased activity of c-src kinase seems not to be an essential step for the signal transduction originating from HER2/Neu oncogene.

On the other hand, our previous observations (Zagozdzon et al., 2002), which did indicate CHK as a possible factor positively influencing process of cancerogenesis. The mechanism of CHK action was attributed to the activation of PI3-K/Akt pathway and could be also ascribed to prolonged stimulation of MAPK kinase pathway. Nevertheless, our experiment did not reveal CHK stimulating oncogenesis in any of the three chosen models. Taken together, our experiments did not find reaffirmation of CHK role in mammary tumor formation.

#### **Discussion:**

There is little known about the role(s) of CHK in mammary physiology or breast cancer. It was shown that CHK is expressed in a variety of mammary cancers, whereas it is not expressed in normal mammary tissues (Bougeret et al., 2001). Most importantly, CHK was induced in mammary cancer cell lines upon heregulin stimulation. Moreover previous data suggested CHK to be a negative regulator of ErbB2 signaling. CHK was found to associate through its SH2 domain with activated ErbB2, and down-regulate activity of ErbB2/Neu-activated src kinases (Zrihan-Licht et al., 1998; Zrihan-Licht et al., 1997). These results indicated that CHK is an important signaling molecule involved in ErbB2 and heregulin signaling.

Recently, SFKs were shown to be an essential step in triggering MAPK signaling upon EGF or heregulin stimulation (Olayioye et al., 2001). Multiple observations support

the role of SFKs as an inevitable step in initiation of the breast cancer as well as important protein for progression of the disease. The first observations indicating that Src kinase activity was higher in mammary neoplastic tissue than in adjacent tissue had been made in early eighties (Jacobs & Rubsamen, 1983; Koster et al., 1991; Lehrer et al., 1989; Rosen et al., 1986). The observations were also confirmed in mammary cancer cell lines derived from transgenic mice, as well as in primary tumors that occurred in those animals (Amundadottir & Leder, 1998; Muthuswamy & Muller, 1995; Muthuswamy et al., 1994). Substantial evidence for essential role in oncogenesis was delivered by studies employing mice expressing Polyoma middle T antigen (PyMT) under breast specific promoter (Guy et al., 1994). Those animals when interbred to c-Src knockout mice and the PyMT(Tg)+Scr(-/-) mice developed tumors less frequently at delayed rate. Moreover, recently c-Src has been shown to associate with ErbB2 (Belsches-Jablonski et al., 2001) in human breast cancer lines. In these studies inhibition of SFKs using PP1 resulted in decreased anchorage independent growth and triggered apoptosis. A similar observation has been made in terms of other ErbB family members e.g. EGFR. It must be pointed out however that, to our best knowledge, the necessity of SFKs activation in Neu-induced breast tumor formation was not confirmed using genetically modified mice model. Therefore, we have decided to use CHK, as a natural inhibitor of Src activity following Neu activation (Bougeret et al., 2001), and to generate transgenic mice expressing CHK in the mammary tissue. As shown in Fig. 5B, we have succeeded to inhibit Neu-induced Src activity in CHK-expressing animals. As a consequence, we expected CHK to inhibit mammary tumor formation or at least slow down growth of Neu-induced tumors. However, in our experiments we did not observed any significant influence of CHK expression on the dynamic of tumor occurrence or growth (Fig. 5A). Moreover, tumors that have developed in any group have comparable levels of PCNA or apoptotic cells within tumor (data not shown). We have not also observed a significant effects of CHK on the Neu-induced transformation of MCF10A when studied in the 3D culture in vitro model (Fig. 4)

Taken together, our results show that downregulation of SFKs activity using CHK overexpression have no significant impact on ErbB2/Neu mediated oncogenesis and did not influenced growth rate of ErbB2-dependent tumors. We find this observation

extremely interesting and warranting further investigation. Currently, we should expect a pure model, adopting knockouts (traditional or conditional) of different SFKs, for example yes, src, fyn crossbred to tumor prone animals expressing Neu, which would finally confirm results indicating role of SFKs in tumorigenesis.

### **Experimental procedures:**

Generation of MMTV-CHK transgenic mice. We used the CHK gene originating from a previously described pcDNA3-based construct (Zrihan-Licht et al., 1998) to generate the MMTV-CHK-SV40 construct. The HindIII restriction site within the CHK gene was deleted (without altering the amino acid sequence) using site-directed mutagenesis and the CHK[HindIII(-)] mutant was cloned between the HindIII and EcoRI restriction sites of the MMTV-SV40-BSSK+ plasmid (generous gift from Dr. Philip Leder). The MMTV-SV40-BSSK construct with the CHK insert was linearized with SalI and SpeI (Figure 1A), resolved on 1% agarose gel, band of CHK-MMTV-SV40 was excised from the gel and the DNA was purified using the Qiaquick gel extraction kit (Qiagen Inc, Valencia, CA). DNA was filtered through a 0.2  $\mu$ m filter, microinjected into pronuclear stage zygotes and transferred into pseudopregnant recipients at the Transgenic Core Facility located at BIDMC. Founders were crossbred to wild-type FVB mice, and strains with germline transmission were bred for further use.

Southern blotting. Tail clips were overnight digested with proteinase K (Roche, Basel, Switzerland). DNA was purified from tail clips using phenol: chloroform extraction method. 10  $\mu$ g of purified DNA was subjected to 16hr digestion with restriction endonucleases EcoRI and Hind III or BamHI (New England Biolabs, Beverly, MA). Digested DNA was resolved on agarose gel, denatured and transferred onto nylon membrane (Hybond N+, Amersham, Piscataway, NJ) and crosslinked. Human CHK cDNA was labeled radioactively using Megaprime Kit (Roche) and  $\gamma$ - $P^{32}$ -dCTP (Perkin Elmer, Boston, MA). Hybridization was carried for 16hr at 55°C in church buffer. After washing membranes were exposed to radiographic film (Kodak, New Haven, CT).

PCR: 2 $\mu$ l purified DNA was subjected to amplification at iCycler (Bio-Rad, Hercules,

CA). 1.2 U of Taq polymerase (New England Biolabs) was used in each reaction. Neu and MMTV primers were obtained from Jackson Laboratories (Bar Harbor, ME) - <http://www.jax.org>. Primers for CHK transgene were design using DNASTar software (DNASTAR Inc., Madison, WI) and obtained from Integrated DNA Technologies (Coralville, IA): CHK-TR4F – 5'-GCC TGC GAG AAC AAG AGC-3', CHK-TR3R – 5'-GCC TCC GAC ACC TCT TTC-3'. Reaction was carried through: 1x 95°C for 30 sec., 36 x (95°C for 30 sec, 63°C for 45 sec., 72°C for 1 min) and final elongation for 7 min.

Histological and morphological (mammary gland whole mounts) evaluation. Complete autopsies were performed and both gross and microscopic examinations.

To evaluate physiological development of the mammary tissue, five mice from each stage (virgin animal – 4 weeks of age; during pregnancy – 16-5 days post coitum; lactating – 1.5 day after delivery; an glands during involution after lactation) from MMTV-CHK transgenic mice as well as the non-transgenic (wt) FVB/N strain were analysed. Inguinal mammary fat pads were excised, fixed in Bouin's solution, blocked in paraffin, sectioned at 5 µm, stained with hematoxylin and eosin, and examined at Rodent Histopathology Core at Harvard Medical School. Mammary whole mount preparations were prepared from inguinal mammary fat pads as described in (Wang et al., 1990). Briefly, inguinal mammary glands were dissected, spread onto glass slides and fixed with a 1:3 mixture of glacial acetic acid: ethanol. After rehydration, whole mounts were stained with 0.2% carmine and 0.5% AIK(SO<sub>4</sub>)<sub>2</sub>, dehydrated and cleared in toluene and methyl salicylate.

Tumor growth analysis. Each experimental group consisted of 25-40 animals. All mice were observed daily for tumor appearance. Tumor free days in each experimental group were counted. To analyze the difference in morbidity between the single and bitransgenic strains, Kaplan-Meyer plots were used. Statistical analysis was assessed with the Mann-Whitney *U* test.

Protein extract preparation. Tissue samples (tumors or mammary glands from control



animals) were fresh frozen in liquid nitrogen and ground to a fine powder with a chilled mortar and pestle. Cells were lysed on ice for 30 min in TNE lysis buffer (20 mM Tris [pH 8.0], 150 mM NaCl, 1% Nonidet P-40, 25 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin) with constant agitation. Lysates were cleared by centrifugation at 13,000 X g for 15 min. Supernatants were collected, protein concentration were determined with the Bradford assay kit (Bio-Rad). Expression of CHK, ErbB2, Src, MAPK, PI3K, and Akt as well as the phosphorylation status of MAPK and Akt in the tissue lysates were assessed by a Western blot procedure using commercially available antibodies.

Src kinase assay. One mg of protein extracts (tumors or mammary glands from control animals) were immunoprecipitated using antibodies against Src (clone GD11, Upstate Biotechnology). The immunoprecipitates were washed 3 times with the lysis buffer and then resuspended in 30 µl kinase buffer containing, 0.25 mg/ml poly(Glu/Tyr)4:1 or acid denatured enolase (both from Sigma) as an exogenous kinase substrate and 10 µCi of [ $\gamma$ - $^{32}$ P]ATP. After 10 min at 30°C, the reaction was stopped by adding SDS-sample buffer, followed by boiling the samples for 10 min. Subsequently, the samples were resolved on 12% polyacrylamide-SDS gels and the gels were stained with Coomassie blue. The labeled poly(Glu/Tyr) lanes were excised from the gel and the radioactivity was counted.

Western Blotting: 60 µg of protein extracts were electrophoretically separated on 10-12.5% polyacrylamide-SDS gels, transferred to nitrocellulose membrane (Bio-Rad) and probed with antibodies against CHK, Csk (Santa Cruz Biotechnology, Santa Cruz, CA), actin (Chemicon International, Temecula, CA),  $\alpha$ -Src (clone GD-11, Upstate, Chicago, IL), Erk1/2 and phospho-Erk1/2 (Cell Signaling Technologies, Beverly, MA)

Cell lines and matrigel cultures: MCF10A cells were kindly provided by Joan S. Bruggie from Harvard Medical School. Cells were maintained in standard mammary medium: DMEM/F12 1:1 media mixture (Invitrogen [Gibco], Carlsbad, CA) supplemented with insulin (Sigma), hydrocortisone (Sigma), azide-free cholera toxin (EMD Biosciences, San Diego, CA), rhEGF (Peprotech, Rocky Hill, NJ), 5% horse serum (Invitrogen Gibco),

antibiotic and L-glutamine (both from Cellgro Mediatech, Herndon, VA ). For matrigel cultures 1000 cells were inlayed onto matrigel (Invitrogen) covered 8-well glass culture chamber slides (Nunc, Lab-Tek, Rochester, NY) and cultured in standard mammary media.

Immunoflorescence staining: All stainings were done according to the protocols by Debnath *et al.* (Debnath et al., 2003a). In all experiments cells were fixed 2% PFA containing phosphatase inhibitors (b-glycerophosphate, sodium orthovanadate, sodium fluoride, all from Sigma). After staining cells were mounted with Vectashield DAPI (Vector Laboratories, Burlingame, CA) and analyzed using a Zeiss LSM 510 Meta confocal microscope at the Harvard Center for Neurodegeneration and Repair Optical Imaging Facility.

Animals: FVB/N-Tg(MMTVneu)202Mul/J (Jackson Laboratories, Bar Harbor, ME), FVB/NJ (Jackson Laboratories), FVB-Tg(MMTV-ErbB2)1Lcd (Charles River Laboratoeis, Wilmington , MA) and FVB-Tg(MMTV-c-myc)1Lcd (Charles River) were used throughout all experiments. All mice were kept under aseptic condition in local animal facility. All procedures were approved by local ethical committee.

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Figure 1.

Generation of MMTV-CHK transgenic mice. (A) Plasmid that was used for microinjection with CHK under MMTV promoter. (B) Southern blot of primary screen of  $F_0$  with CHK probe. (C) Southern blot of  $F_1$  transgenic mice with CHK probe. (D) Genotyping of double transgenic mice. (E) Western blot of transgene (CHK) and endogenous Ctk expression in different tissues of MMTV-CHK mice. (F) Expression of transgene (CHK) in mammary tissue of all strains of MMTV-CHK mice.

Figure 2.

Development of mammary tree in MMTV-CHK transgenic mice vs. wild-type animals. (please see Experimental Procedures for details).

Figure 3.

(A) c-Src activity in mammary glands of MMTV-CHK transgenic animals. (B) Phosphorylation of p42/p44 MAPK in mammary glands of wild-type, MMTV-CHK and MMTV-Neu animals.

Figure 4.

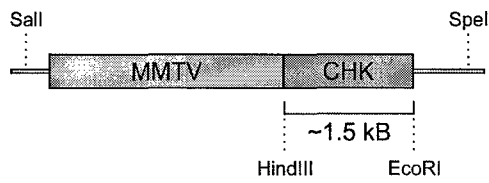
Three-dimensional cultures of MCF10A cells transduced with retroviruses encoding CHK262 (dead kinase) or CHK wt. (please see Experimental Procedures for details).

Figure 5.

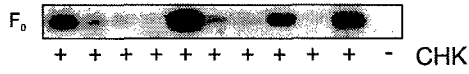
(A) Mammary tumor occurrence in single (activated NeuT or non-activated Neu) expressing mice vs. double-transgenics (mice expressing NeuT and CHK or Neu and CHK in mammary glands). (B) c-Src activity in mammary glands and tumors in single- and double-transgenic mice.

**Figure 1**

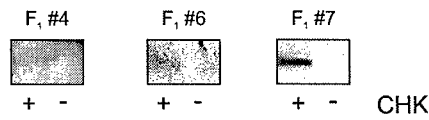
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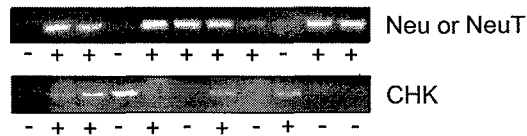
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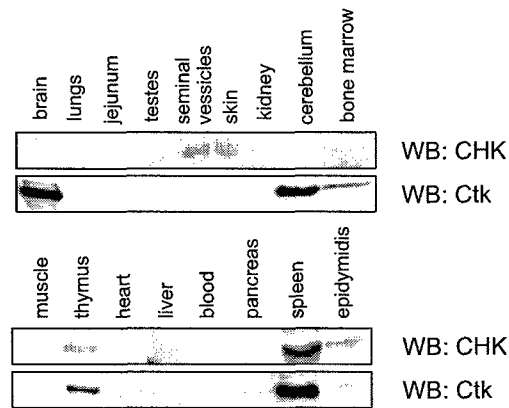
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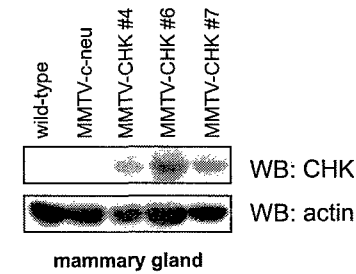
**D.**



**E.**

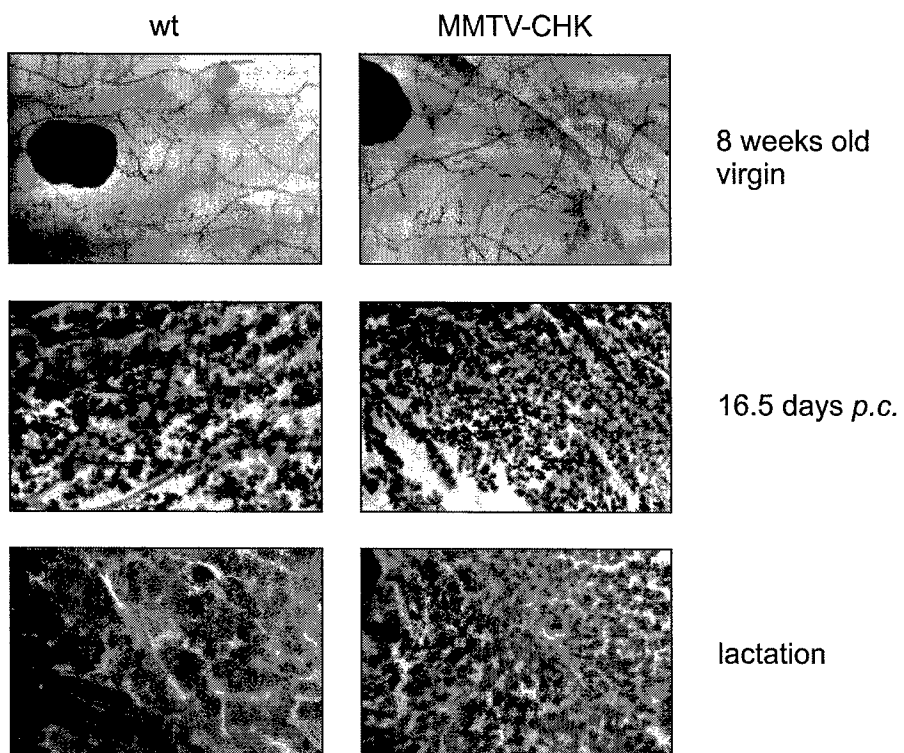


**F.**



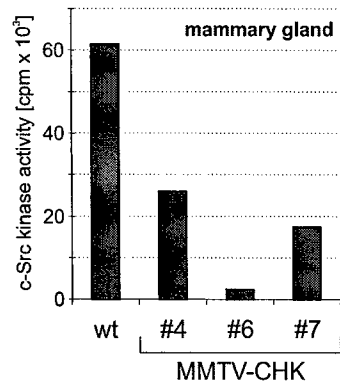


**Figure 2**

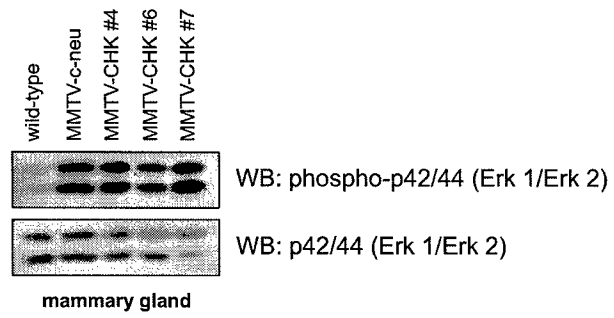


**Figure 3**

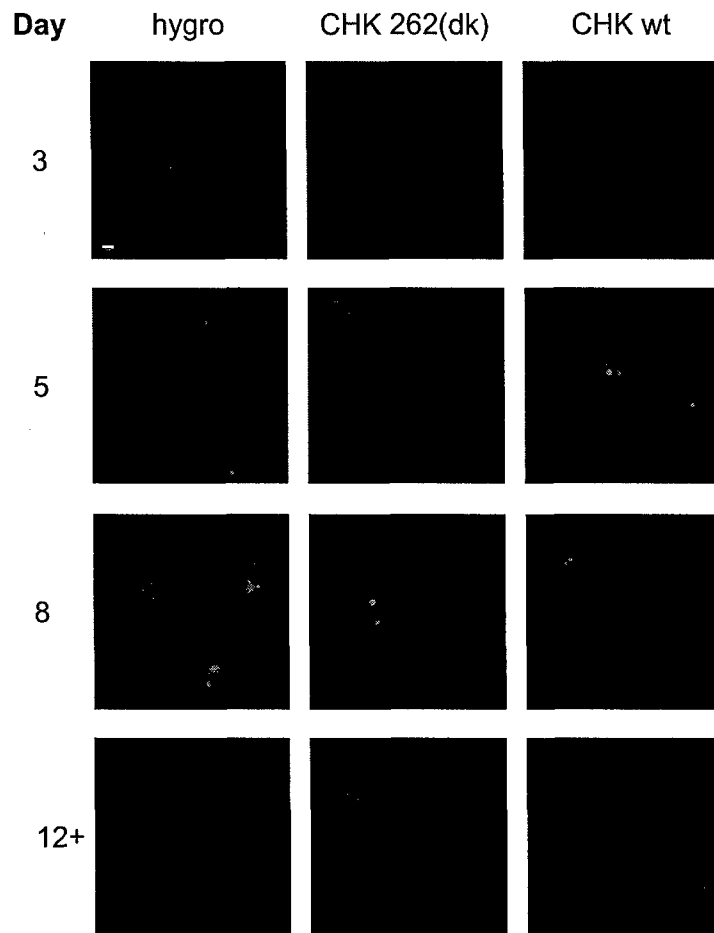
**A.**



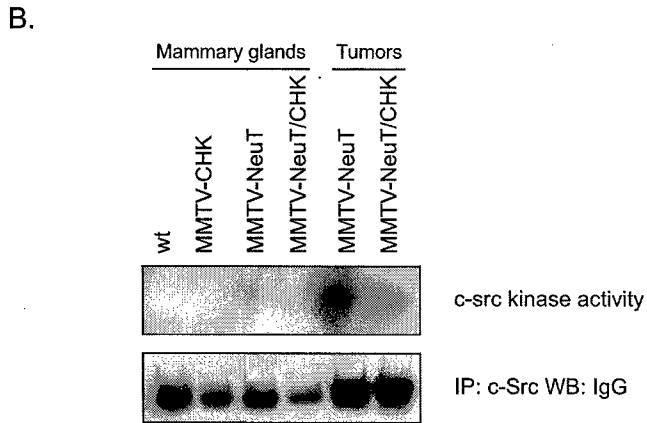
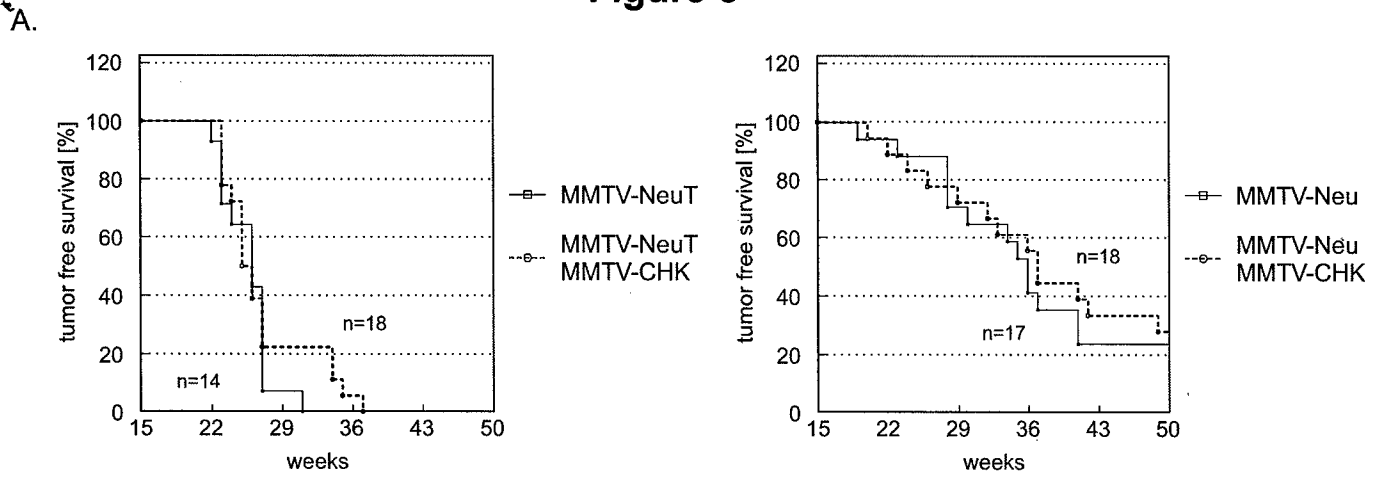
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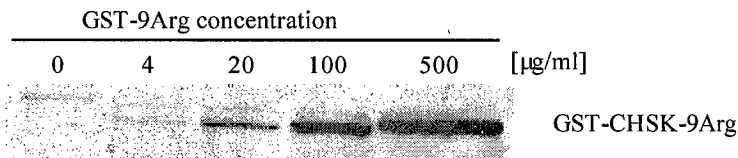
**Figure 4**



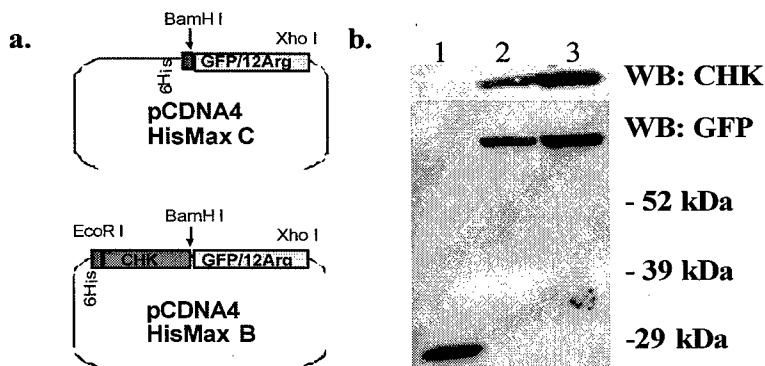
# Figure 5



## Attachment #6



**Figure 1: Uptake of GST-9Arg fusion protein from culture medium.** Eighty-percent confluent T47D cells were incubated with the various concentrations of GST-9Arg for 30 min. The cells were thoroughly washed in PBS, lysed in protein lysis buffer and then SDS/PAGE plus Western blot analysis was performed with anti-GST antibodies.



**Figure 2: Generation of vectors encoding proteins fused with polyarginine.**

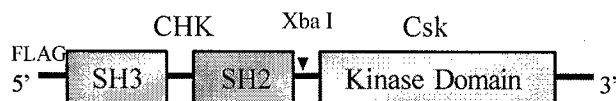
**a.** Maps of mammalian expression vectors generated to express either GFP-12Arg or CHKwt/dk-GFP-12Arg fusion proteins.

**b.** Western blot analysis of the expression of GFP-12Arg (Lane 1), CHKwt-GFP-12Arg (Lane 2), and CHKdk-GFP-12Arg (Lane 3) proteins in CHO cells.

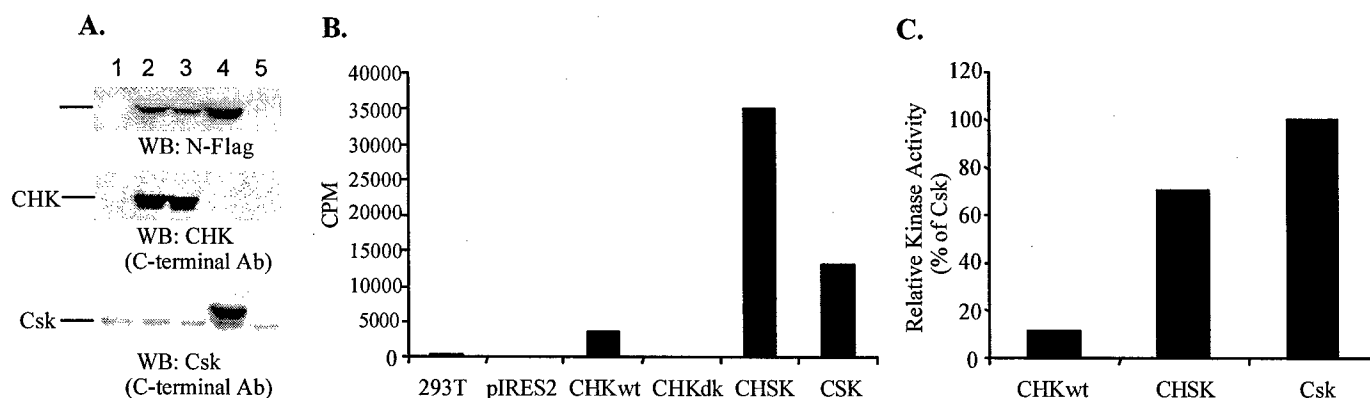


**Figure 3: Transient expression of GFP-12Arg and CHK-GFP-12Arg fusion proteins in CHO cells.**

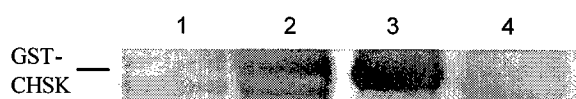
The cells were seeded onto a 6-well plate, grown until 80% confluent, and then transfected with 3 µg of GFP-12Arg/pcDNA4HisMaxC (A), CHKdk-GFP-12Arg/pcDNA4HisMaxB (B) or CHKwt-GFP-12Arg/pcDNA4HisMaxB (C) using Lipofectamine 2000 reagent. Fluorescence was assessed 24 h following transfection. Magnification 400x.



**Figure 4: Schematic presentation of CHK/Csk (CHSK) chimeric fusion cDNA.**



**Figure 5: A. Expression of CHK(wt), CHK(dk), and CHSK fusion proteins in 293T cells, as compared to endogenous Csk.** 293T cells were seeded onto 6-well plates, grown until 80% confluent, and then untransfected or transfected with 3  $\mu$ g of empty vector (Lane 1), CHK(wt) (Lane 2), CHK(dk) (Lane 3), CHSK (Lane 4) in pIRES2-EGFP using Lipofectamine 2000 reagent. (Lane 5=untransfected cells). Expression of CHK (wt), CHK(dk), and CHSK as well as endogenous Csk was evaluated by Western blotting using specific antibodies. **B. Tyrosine kinase activity of CHK(wt), CHK(dk), CHSK following transient transformation of 293T cells as compared to endogenous Csk.** **C. Relative tyrosine kinase activity of CHK(wt) versus CHSK as a percentage of Csk kinase activity.** Cpm reading was normalized for densitometry analysis (Scion Image for Windows software, NIH).



**Figure 6: Expression in *E. coli* and binding of GST-CHSK fusion protein to glutathione-Sepharose beads.** Lanes: 1 – lysate of non-induced bacteria, 2 – lysate of induced bacteria, 3 – GST-CHSK bound to the beads, 4 – lysate of induced bacteria following incubation with the beads (supernatant)



**Figure 7. GST pull-down experiment.** Serum-starved MCF7 cells were stimulated with 20 nM heregulin for 8 min at room temperature and then lysed in protein lysis buffer. Lysates were incubated with GST fusion proteins coupled to glutathione-Sepharose beads. The precipitated proteins were developed on an SDS-7% polyacrylamide gel and then transferred onto nitrocellulose membrane. Bound proteins were immunoblotted with anti-phospho-HER2/ErbB-2 (Tyr1248) antibody. Lanes: 1 – GST alone, 2 – GST-CHK(SH2), 3 – GST-CHSK.